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Management and Genetic Variability of Kikuyugrass (*Pennisetum clandestinum* Hochst. Ex Chiov.)

A Dissertation submitted in partial satisfaction
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by

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ABSTRACT OF THE DISSERTATION

Management and Genetic Variability of Kikuyugrass (*Pennisetum clandestinum* Hochst. Ex Chiov.)

by

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Doctor of Philosophy, Graduate Program in Plant Biology
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Dr. James H. Baird, Chairperson

Kikuyugrass is an invasive, warm-season turfgrass that was introduced into California in the 1920s. Although it is used in many sport and residential turfgrass areas, very little focus has been on the improvement of the species. A comprehensive project was designed and implemented to address kikuyugrass management, improvement through anther culture, and genetic variation within California and other collections.

A field trial was conducted in 2012 and 2013 to identify cultural and chemical practices that are most important for producing quality turf and optimal playing conditions on golf course fairways. A 2^5 factorial design was used to evaluate mowing frequency, cultivation, trinexapac-ethyl, nitrogen, and fungicide treatments. Turf quality was assessed visually and by an instrument that measures "greenness" or Normalized Difference Vegetation Index (NDVI). Turf firmness and ball roll were measured with a Clegg Soil Impact Tester (2.5 kg hammer G_{max}) and Pelz meter, respectively. Treatments of trinexapac-ethyl, aggressive verticutting, and more frequent mowing practices produced the highest quality turfgrass by increasing firmness, improving turf quality and color, and reducing scalping.

Androgenesis through anther culture was attempted on kikuyugrass in order to develop a less vigorous cultivar. Androgenesis has been successful on the closely related pearl millet (*Pennisetum glaucum*) but never attempted on kikuyugrass. Kikuyugrass and later pearl millet were tested with two types of media (C17 and Yu-Pei) combined with two different incubation regimes. No signs of mitosis were seen on any of the observed anthers and no embryos were formed in either species. The results were inconclusive and the possibility of successful androgenesis in kikuyugrass remains an open question.

To assess the presence of genetic variation as an indispensable condition for eventual breeding efforts, genotypes were sampled from collections in California (from around the state and at the University of California, Riverside), Hawaii, and Australia. A total of 20,000 single nucleotide polymorphism (SNP) makers were discovered using the Diversity Array Technology sequencing (DArTseq) platform. The hierarchical plot, gap statistics, and principle coordinate analysis all showed that the 336 accessions in the study separated into three main clusters. The analysis showed that the amount of genetic variation was low, with 77% of the total genetic variation due to within population variation, while 23% was due to among population variation. The accessions from California showed the least genetic variation while accessions from Australia and Hawaii showed a much broader range of genetic diversity. Although California kikuyugrass accessions showed low genetic diversity, there may be enough variation among the accessions at the University of California, Riverside and those collected in the state to begin breeding a turf-type cultivar in California.

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Chapter 1

General Introduction

Kikuyugrass is a warm-season turfgrass species that originated from the east African Highlands and now inhabits every continent except Antarctica (Mears, 1970). It was first imported into the United States in the 1920s (Garner, 1925) with the intent to utilize it for soil erosion control on hillsides and riverbanks; however, it quickly spread to colonize much of southern and central California. Today, kikuyugrass is considered an invasive weed whereby sale and transport are prohibited in several California counties. Furthermore, it is on the Federal Noxious Weed list, which restricts importation of samples into the country and across state boundaries (USDA, 2012).

Kikuyugrass spreads aggressively through rhizomes, stolons, and seed (Youngner et al., 1971). Also found in Arizona and Hawaii, the species is well-suited to Mediterranean climates like California because it can photosynthesize across a wide temperature range as evidenced by its superior winter color retention among the warm-season turfgrasses (Wilen and Holt, 1995). These characteristics have allowed kikuyugrass to invade many areas including golf courses, athletic fields, and lawns, where it often becomes the dominant turfgrass species. Rather than attempting to selectively remove kikuyugrass, some landscape managers attempt to utilize it (Gross, 2003).

Challenges in managing kikuyugrass include: aggressive growth, sensitivity to many selective herbicides, and low shade tolerance (Gross, 2003). Aggressive growth of kikuyugrass, especially under adequate water and fertilization, leads to substantial

scalping, making the turf susceptible to pathogens and aesthetically unpleasing (Beard, 1973). All kikuyugrass turf, regardless of mowing height, is susceptible to scalping. Kikuyugrass management strategies to produce a high quality turf stand often require a combination of cultural and chemical practices to counteract aggressive growth and scalping that is typical in spring and summer. These include: verticutting, frequent mowing, application of plant growth regulators (PGRs), limited nitrogen fertilization, and fungicides for control of major kikuyugrass diseases including Rhizoctonia large patch, gray leaf spot, and decline (Gross, 2003).

Kikuyugrass is a tetraploid with a somatic chromosome number of 2n= 36. It is an outcrossing species with several bisexual spikelets per inflorescence (Mears, 1970). Although there has been some research on improvement of kikuyugrass as a forage in other countries, relatively little research has been done on kikuyugrass improvement for turf. There are only two cultivars (Whittet and AZ-1), both of which are forage types, which are propagated in California. Due to restriction on germplasm exchange because of the noxious weed status, it is vital to try to utilize and improve the genetic resources available in California to create a turf-type cultivar.

A major problem with kikuyugrass is its aggressive growth habit. A potential mechanism to reduce this growth habit is through ploidy reduction. Ploidy manipulation to develop new traits is common in domesticated plants. Triploidy is used in watermelon and bananas to produce seedless varieties (Acquaah, 2007). Polyploids created through chromosome doubling are often desirable for their increased cell size and enlarged plant

organs (Emsweller and Ruttle, 1941; Schepper et al., 2001). Ploidy reduction is a tool that could be used to reduce vigor, and it can be achieved through androgenesis.

Androgenesis is the process of induction and regeneration of haploids and double haploids from gametic cells. It was first developed in *Datura inoxia* when Guha and Maheshwari (1964) discovered that immature pollen could be induced to bypass normal gametophytic development within the anther to produce haploid plants. Reduction in ploidy through androgenesis has been successful in pearl millet and maize, both of which are closely related to kikuyugrass (Nitsch et al., 1982; Budak et al., 2004). It has also been successful in the turfgrass species *Lolium perenne* and *Festuca pratensis* (Kopecky et al., 2005). Usually androgenesis is used to create double haploid plants (Sood and Dwivedi, 2015). Chromosome doubling is usually associated with increased plant size. The opposite effect could be expected for haploid production, and has been recorded in pepper, *L. perenne*, and *F. pratensis* (Dolcet-Sanjuan et al., 1997; Kopecky et al., 2005).

Another method of kikuyugrass improvement is standard breeding approach: intercrossing of genotypes with desirable turf-type phenotypes and progeny selection. Due to its plastic nature it is difficult to distinguish among different kikuyugrass genotypes in residential areas and on heavily managed sports turf like golf course fairways (Morris, 2009). Therefore, the genetic diversity within CA needs to be studied to determine if there is a sufficient range of variation to breed a turf-type cultivar.

Genetic variation is vital for breeding success. No progress of selection can be expected in homozygous material. Only one genetic variation study was conducted on kikuyugrass in California. Using isozymes Wilen *et al*. (1995) found very little genetic

diversity among subpopulations at three different golf courses meant to represent northern, central, and southern California. Another study in Australia used DNA amplified fingerprinting (DAF) and 40 polymorphic loci to identify two main groups among 13 genotypes (Holten *et al*., 2007). Morris (2009), using random amplified polymorphic DNA (RAPD) found three main groups among 19 genotypes using 195 markers and concluded that significant variation did exist within Australian genotypes.

There are two potential sources of genetic variation available in CA. Such variation may exist among genotypes throughout the state because of genetic differences among different introgressions, or different accumulation of mutations. Genetic variation may also be present in kikuyugrass accessions maintained at the University of California, Riverside (UCR). Twenty such accessions were developed by Dr. Ruth Shaw from 1990-1992 (Cockerham *et al*., 1992) following selection from a single bag of kikuyugrass seed imported from Australia. Selection was based on winter color retention, growth habit, sward density, leaf texture, and spread. Cockerham et al. (1992) described significant differences among accessions. Samples from collections in Hawaii and Australia were included in the study in case results showed low genetic diversity among California genotypes.

Genetic diversity analysis can be accomplished through an analysis of single nucleotide polymorphisms (SNP) detected using the Diversity Array Technology sequencing (DArTseq) platform. The next generation sequencing (NGS) combined with the existing DArT marker platform can produce a rapid single nucleotide polymorphism (SNP) discovery method (Sansaloni *et al*., 2011; Kilian *et al*., 2012; Courtois *et al*., 2013;

Cruz *et al*., 2013; Raman *et al*., 2014). DArTseq uses restriction enzymes to separate low copy sequences from the repetitive fraction of the genome and represents a new implementation of complexity-reduced representations (Altschuler *et al*., 2000). The main reason for using DArTseq is its ability for SNP discovery in species with no previous DNA sequencing information. It is also capable of generating in a single step thousands of markers for genetic diversity analysis as compared to the hundreds developed with all previous molecular methods combined. It has already been used to successfully assess genetic diversity in Lesquerella (*Physaria sp*.) (Cruz *et al*., 2013).

This body of work explores three directions towards kikuyugrass improvement focused on both short-term immediate applications as well as long-term improvement of the species.

Objective 1. Determine which combination of management practices work best for producing high quality kikuyugrass turf.

Objective 2. Determine if a reduction of vigorous growth in kikuyugrass is possible via ploidy reduction.

Objective 3. Determine if enough genetic variation exists within California kikuyugrass germplasm to initiate breeding for a turf-type cultivar.

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Chapter 2

Cultural and Chemical Management Practices for Optimal Kikuyugrass Quality and Playing Conditions

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Abbreviations: NDVI, Normalized Difference Vegetation Index; PGR, plant growth regulator; TE, trinexapac-ethyl; TQ, turf quality; UCR, University of California, Riverside;

ABSTRACT

Kikuyugrass (*Pennisetum clandestinum* Hochst. ex Chiov.) is considered either an invasive weed or the desired species on many golf courses and other turf areas along coastal and inland California. A field study was conducted in Riverside, CA in 2012 and 2013 on 'Whittet' kikuyugrass mowed at 1.1 cm to identify cultural and chemical practices important for producing good quality turf and optimal playing conditions for golf course fairways. A two-level, five-factor factorial design was used to evaluate mowing frequency, cultivation, a plant growth regulator (trinexapac-ethyl), nitrogen fertilization and fungicide treatment. Turf quality was assessed visually and by Normalized Difference Vegetation Index (NDVI). Turf firmness, ball roll distance, and tensile strength were also measured. Trinexapac-ethyl (TE), verticutting twice yr^{-1}, and mowing six times yr^{-1} produced the highest quality turf and least scalping during summers of 2012 and 2013 when kikuyugrass grows most vigorously. In general, verticutting twice yr^{-1} without TE produced the firmest turf, and ball roll was increased with grooming compared to verticutting. These results demonstrate that kikuyugrass available in California requires maximal inputs for optimal quality and playability. Future breeding efforts in California should focus on developing improved cultivars with reduced cultural and chemical inputs.

Kikuyugrass is a warm-season turfgrass species that originated from the east African Highlands and now inhabits every continent except Antarctica (Mears, 1970). It was first imported into the United States in the 1920s (Garner, 1925) to utilize it for soil erosion control on hillsides and riverbanks; however, it quickly spread to colonize much of southern and central California. Today, kikuyugrass is officially considered as an invasive weed with sale and transport prohibited in several California counties. Furthermore, it is on the Federal Noxious Weed list, which restricts importation of germplasm into the country and across state boundaries (USDA, 2012).

Kikuyugrass spreads aggressively through rhizomes, stolons, and seed (Youngner et al., 1971). Also found in Arizona and Hawaii, the species is well-suited to Mediterranean climates like California because it can photosynthesize across a wide temperature range as evidenced by its superior winter color retention among the warm-season turfgrasses (Wilen and Holt, 1995). These characteristics have allowed kikuyugrass to invade areas including golf courses, athletic fields, and lawns, where it often becomes the dominant managed turfgrass species rather than attempts to selectively remove it (Gross, 2003).

Challenges in managing kikuyugrass include aggressive growth, intolerance to many selective herbicides, and low shade adaptation (Gross, 2003). However, intolerance to herbicides does not translate to easy kikuyugrass eradication. Chlorosis generally follows herbicide applications then after some time, depending on the herbicide, the grass recovers. Aggressive growth, especially under adequate irrigation and fertilization, leads to substantial scalping, making the turf susceptible to pathogens

and aesthetically unpleasing (Beard, 1973). All kikuyugrass turf, regardless of mowing height, is susceptible to scalping. Management strategies to produce a high quality turf stand often require a combination of cultural and chemical practices to counteract aggressive growth and scalping that is typical in spring and summer. These include: verticutting, frequent mowing, plant growth regulators (PGRs), limited nitrogen fertilization, and fungicides for control of major diseases such as Rhizoctonia large patch, gray leaf spot, and decline (Gross, 2003). Research on kikuyugrass cultural practices is limited. Cockerham et al. (1998) studied verticutting on and found that it reduced organic matter and increased turfgrass firmness. They concluded that verticutting more than three times yr^{-1} was unnecessary on heavily trafficked areas.

Mowing frequency in combination with PGRs has been studied in both warm- and cool-season grasses. Johnson (1994) found less mowing was required with the use of trinexapac-ethyl (TE) on bermudagrass (*Cynodon dactylon* (L.) Pers.), but the research did not address the effects of mowing frequency. Jagschitz (1982) found that the PGR mefluidide [N-[2,4-Dimethyl-5-[[(trifluoromethyl)sulfonyl]amino]phenyl] acetamide] reduced overall mowing treatments needed to maintain stands of Kentucky bluegrass (*Poa pratensis* L.). Mowing frequency influenced the performance of PGRs mefluidide and Mon 4620 [N-[(acetylamino)-methyl]-2-chloro-N 2,6(diethylphenyl)acetamide] on vegetative growth and seed head control but not plant injury, quality, or shoot density (Johnson, 1989).

Trinexapac-ethyl is commonly used on turfgrass to increase visual quality, color, and shoot density (Qian and Engelke, 1999; Ervin and Zhang, 2008; Kreuser and Soldat,

2011). It has also been used on warm-season grasses to reduce scalping (Totten et al., 2006). Stowell and Gelernter (2001) found that applications of 0.032 kg a.i. ha^{-1} of trinexapac-ethyl caused significant reduction in scalping and clipping yield of kikuyugrass. This rate also provided the best turf quality when compared to 0.064 kg a.i. ha^{-1} or 0.016 kg a.i. ha^{-1} of TE.

Kikuyugrass generally requires low N fertilization in sports turf settings to offset organic matter or thatch accumulation. Barton et al. (2009) investigated multiple rates of N application on kikuyugrass ranging from 50 kg ha^{-1} yr^{-1} to 150 kg ha^{-1} yr^{-1}. They found that more frequent N applications improved consistency, growth, and color. Barton et al. (2009) also found that the quality of mature kikuyugrass stands can be maintained using less N fertilizer than younger stands. Acceptable turfgrass quality was achieved with applications as low as 12 kg N ha^{-1} yr^{-1} on untrafficked turf. Cockerham et al. (1998a) reported that N increased biomass (i.e., thatch) accumulation on kikuyugrass turfgrass stands, especially if N was applied infrequently in smaller increments.

A majority of kikuyugrass management studies has been directed toward its use as a forage species and to a lesser extent for potential uses in bioremediation. Muscolo et al. (2003 and 2012) studied the mechanisms of salinity tolerance to determine its efficacy as a forage or turfgrass on saline soils, finding that it has good salinity tolerance and is better suited for bioremediation of saline soils than most broadleaf species.

The aggressive growth habit of kikuyugrass makes it difficult to manage and little research has been conducted on turfgrass management strategies, and their interactions, for producing high quality turf with minimal inputs. The objective of this research was to

identify management practices and combinations that work best to produce high quality kikuyugrass with minimal scalping for golf course fairways and related uses.

MATERIALS AND METHODS

A field study was conducted at the University of California, Riverside (UCR) Turfgrass Research Facility from April to November 2012 and 2013. The cultivar Whittet was established from sod in July 2011 on a Hanford fine sandy loam (70.4% sand, 19.8% silt, 9.8% clay, 0.6% organic matter, superactive, nonacid, thermic Typic Xerorthents). Initial soil tests revealed sufficient levels of all essential nutrients. Once established, turf was maintained at a 1.1-cm height of cut three times wk^{-1} during the growing season using a 66-cm wide walk-behind-reel mower (Baroness Model LM66T, Richmond, CA). Irrigation was at 80% ET_o of historical monthly cumulative reference evapotranspiration (ET_o) obtained from an on-site California Irrigation Management Information System (CIMIS) weather station that was based on a modified Penman equation with a wind function (Doorenbos and Pruitt, 1984). The CIMIS reference crop was well-watered tall fescue (*Festuca arundinacea* Schreb.) mowed at 7.6 cm. Climatic data for the study period are shown in Table 2.1.

The study was arranged in a 2^5 factorial design with 12 main plots (6 m^2) each containing 16 1.5-m^2 sub-plots. Main plots were replicated three times and were subject to mowing and cultivation treatments. Sub-plots were replicated two times in each main plot and received growth regulator, fertilizer, and fungicide treatments. Overall, each combination of the five factors was replicated six times.

Treatments

Treatments were applied from April to November in each year except for fungicides, which were applied monthly throughout both years. The treatment levels, two per treatment, were designed to mimic either those golf courses with very high kikuyugrass quality and intensive cultural practices, or to simulate less intensive, low maintenance cultural practices. Turf was mowed either 3 or 6 times wk^{-1} in alternating directions. Cultivation included either less aggressive grooming or more aggressive verticutting to remove organic matter. Grooming was performed 3 times wk^{-1} using the groomer attachment on the Baroness mower at the lowest setting of zero. Verticutting was performed twice yr^{-1} using a Ryan Mataway walk-behind machine (Johnson Creek, WI). Plots were verticut June 4 and Aug. 4 in 2012 and June 4 and Aug. 2 in 2013. Trinexapac-ethyl (Primo Maxx, Syngenta Crop Protection, Greensboro, NC) was applied biweekly at either 0 or 0.032 kg a.i. ha^{-1}. Nitrogen was applied as liquid NH_4SO_4 (21N-0P-0K) at either 98 or 244 kg N ha^{-1} yr^{-2} in 24 kg N ha^{-1} increments. The lower N treatments were applied in April, June, Aug., and Oct., whereas the higher treatments were applied every 21 d. Fungicide treatments were applied monthly in comparison to no fungicide treatment. Application rates and timings of fungicide treatments are provided in Table 2.2. Fungicides, TE, and N were applied using a CO_2-backpack sprayer calibrated to deliver 814 L ha^{-1}.

Ratings

Ratings were taken at the beginning of each month from May-November 2012 and 2013. The monthly ratings were averaged and analyzed as three different seasons

(May-June, July-Sep., and Oct.-Nov.). Turf quality (TQ) and scalping were assessed visually. Turf quality was rated on the 1-9 scale (1 = worst, completely brown and desiccated and 9 = best, green, dense, turgid canopy; 6= minimally acceptable). Scalping was rated as a percent of total area. Turf color was measured by the Normalized Difference Vegetation Index (NDVI) using a Field Scout CM-1000 chlorophyll meter (Spectrum Technologies, Plainfield, IL). NDVI instruments have been used to determine canopy green leaf biomass, and chlorophyll content in bentgrasses (*Agrostis spp*.) (DaCosta and Huang, 2006). Ball roll distance was measured using a PELZmeter™ (PelzGolf Inc., Spicewood, TX). This rating was included because kikuyugrass canopy architecture is known to impede golf ball roll on fairways.

Turf firmness was measured in G_{max} with a 2.25 kg hammer Clegg Impact Soil Tester (CIST/882/2.25kg; SD Instrumentation Ltd., Top Farm Tellisford Bath, England). The Impact Soil Tester, which will be referred to hereafter as "Clegg" uses a decelerometer to gauge the hardness or surface impact resistance of a sports turf (Clegg, 1976). Three Clegg Soil Impact Tester ratings were taken for each subplot. The average of each subplot was taken before the data were analyzed. McAuliffe (2008) suggested the ideal Clegg measurement for turfgrass to be between 70-89 G_{max} with the lower range being between 31-69 G_{max}.

Footing or traction was determined using a traction plate, which is a cleated, 42-kg steel plate dropped into the turf which is meant to represent a cleated foot. The traction plate was developed and improved by Canaway and Bell (1986) to measure the amount of power it takes to break or tear a turfgrass. The force required to turn the plate

in turf is recorded as meter-kilogram torque (m-kg) with a torque wrench. McAuliffe (2008) suggested 4-5.5 kg/m for ideal traction strength.

Statistics

Data for all monthly ratings were subjected to analysis of variance as a 2^5 factorial with a split plot design with six replications. Monthly ratings were averaged and analyzed as three different seasons May-June (spring), July-Sep. (summer), and Oct.-Nov. (fall). An ANOVA testing the interactions and main effects of five experimental factors on six variables (TQ, NDVI, Scalping, BRD, Clegg, and Tension) was run for each season. Means were separated using Fisher's protected least significant difference ($p \leq 0.05$). Data analysis was performed using Statistix 8 (version 8.0; Analytical Software, Tallahassee, FL).

RESULTS

There were significant differences in ratings between years despite relatively few differences in weather conditions (Table 2.1), thus data are presented separately for spring, summer, and fall of 2012 and 2013 (Tables 2.3-2.5). The data were separated into three seasons to summarize the trends. Spring ratings (May and June) did not include the verticutting treatment, which began in June. Kikuyugrass grows most aggressively, and is most intensely managed, in the summer months. This is also the time when the most scalping can occur. For these reasons, summer months (July, Aug., Sept.) were combined and receive the most detailed discussion. Fall ratings (Oct. and Nov.) represent turf response prior to dormancy.

Trinexapac-ethyl

Overall across seasons and years, TE produced the most effects for the measured variables (Tables 2.3-2.5). In spring 2012, TE generally had positive effects on ratings for TQ, NDVI, scalping, and ball roll distance (BRD). Although the opposite was found for TQ, NDVI, and scalping in spring 2013, the differences were very small and are probably not biologically significant (Table 2.6). Trinexapac-ethyl application did result in less firm turf and weaker tensile strength in both years (data not shown). Fall ratings showed the same pattern as spring. Turf quality and NDVI were reduced slightly and scalping increased slightly in fall 2012 (Table 2.6); whereas, larger and positive effects for these variables were observed in fall 2013 (data not shown).

A similar pattern in ratings was observed in summer of both years but with more pronounced effects. The application of TE produced significantly higher TQ and NDVI during summer months. Turf quality means in 2012 for TE and no TE were 6.1 and 5.5, respectively while NDVI means for TE and no TE were 0.76 and 0.72, respectively (data not shown). Trinexapac-ethyl also significantly decreased scalping in summer 2012 (Table 2.7). The same effects on TQ, NDVI and scalping were found in summer 2013 (Tables 2.8 and 2.9). In both years, TE decreased firmness and tensile strength while increasing BRD (Table 2.10).

Cultivation

Cultivation was another factor that had significant effects on measured variables. In spring 2012, only grooming had been initiated with verticutting not starting until the summer. Grooming had small but significant negative effects on TQ and NDVI (data not

shown). However, the initial grooming treatment resulted in firmer turf and greater BRD and tensile strength. In spring 2013 the effects of verticutting resulted in inferior TQ and NDVI relative to grooming (Table 2.11). However the verticutting in 2012 had a carry-over effect and resulted in firmer turf in 2013 (Table 2.11).

In contrast to spring 2012 and 2013, verticutting in summer and fall of both years showed increased TQ, NDVI, firmness, and tensile strength and decreased scalping relative to grooming (Tables 2.10-2.12). On the other hand, grooming resulted in increased BRD (Table 2.10).

Mowing

Mowing frequency had mixed results for measured variables both within seasons and among years. In general, mowing six times wk^{-1} increased TQ, NDVI, BRD, and firmness. Spring of both years showed increased BRD and firmness with mowing 6 times wk^{-1} when compared to mowing 3 times wk^{-1} (data not shown). Mowing frequency resulted in few significant differences in fall of both years. Many of the significant differences in mowing frequency were seen in summer 2012. Higher mowing frequency resulted in higher TQ and NDVI ratings (Table 2.13), less scalping (Table 2.7) and greater firmness, BRD, and tensile strength (Table 2.10).

Nitrogen

Effects of N were minimal in spring 2012 because little N had been applied at the start of the experiment. In spring 2013, the higher N rate increased NDVI and scalping with higher and lower rates showing 0.74 and 0.73 and 1.2 and 1.8 percent, respectively. Although these differences were statistically significant it is unlikely that they are

biologically meaningful. Effects of N in summer 2012 were also minimal. In 2013, the higher N rate increased TQ, NDVI, scalping, BRD, and decreased firmness and tension. Again, the differences in ratings between the two N rates were very small.

In fall of both years, higher N rates produced better TQ and NDVI. Turf quality ratings for the high and low N rate for 2012 and 2013 were 5.6 and 5.3, and 6.4 and 6 respectively. NDVI ratings for the same parameters were 0.75 and 0.72, and 0.77 and 0.72 respectively. Reduced scalping was seen with the high N rate only in fall 2012 with high and low rate showing 5.5% and 8% respectively. In fall 2013, higher N decreased firmness and tensile strength but the difference was small and probably not biologically significant (data not shown).

Fungicides

Disease pressure was absent during the study period with the exception of a moderate outbreak of large patch (*Rhizoctonia solani* AG2-2) in winter 2012/2013. This resulted in higher TQ and NDVI ratings for spring and summer 2013 for plots treated with fungicides. The differences in both seasons between treated and untreated plots were significant but small and may not be biologically significant (data not shown). Furthermore, increased firmness was noted in response to fungicide applications between summer 2012 and spring 2013 (Table 2.11).

Multi-factor interactions

Most of the tables discussed show two- or three-way interactions. Some of these interactions really only show one treatment factor as dominant and driving the separation into significant homogenous groups (Tables 2.8, 2.9, and 2.12). However, most of the

other tables show that a combination of factors produced significantly better ratings. Table 2.10 shows that the higher mowing frequency, TE, and grooming resulted in the longest BRD in summer 2012. Table 2.11 shows that in fall 2012 and spring 2013 the combination of more frequent mowing, fungicides, and verticutting resulted in firmer turfgrass and reduced scalping. Lastly, Table 2.13 shows that in summer 2012, more frequent mowing combined with verticutting resulted in significantly higher TQ and NDVI ratings.

DISCUSSION

Trinexapac-ethyl

Trinexapac-ethyl and cultivation appear to be among the most important factors for kikuyugrass quality and playability. Trinexapac-ethyl treatments produced both negative and positive effects on kikuyugrass. Reduced scalping with TE applications was seen in summer of both years. This supports previous research showing that TE decreases shoot growth of turfgrasses (Ervin et al., 2002), which should lead to reduced scalping in an aggressive species like kikuyugrass. Higher TQ and NDVI ratings seen in summer of both years with TE applications were most likely due to reduced scalping and darker green color. A study done by Bunnell et al. (2005) showed increased green color on bermudagrass with TE applications and an increase in the chlorophyll concentrations in the shoots. They suggest that increased chlorophyll in the shoots was due to reduced cell and shoot elongation. It is likely that the same effects resulted in darker greener color in kikuyugrass.

Reduction of firmness in kikuyugrass due to TE was likely due to increased lateral growth which is often associated with TE treatments on C4 grasses. Menchyk et al. (2012) found that combined applications of N with TE reduced firmness on zoysiagrass (*Zoysia spp*.) putting greens. Reduced firmness can lead to less ball roll distance from a golf shot or perhaps less secure footing on an athletic field (McAuliffe, 2008). Reduction in tension by TE may be associated with the increased lateral growth and could lead to less secure footing with a cleated shoe. Overall, TE is a very important management tool and should be implemented on managed kikuyugrass wherever possible.

Cultivation

Cultivation is also very important to maintain high quality kikuyugrass. Although verticutting was more aggressive and reduced aesthetics directly after implementation, overall it was found to be a better cultivation practice than the less destructive grooming treatment. Verticutting resulted in higher TQ and NDVI ratings in 2012 most likely due to reduced scalping. In 2013, TQ and NDVI results were more variable in response to the cultivation treatments. Grooming resulted in longer BRD in both years probably due to its less destructive removal of organic material as compared to verticutting. Firmer turf caused by the verticutting treatment was likely due to the greater removal of organic material, even though there were only two verticutting events yr^{-1}. These results support previous research by Cockerham et al. (1998b) which showed that verticutting 2-3 times yr^{-1} was sufficient to maintain high quality kikuyugrass.

Mowing

It was originally thought that the higher mowing frequency of 6 times wk^{-1} would contribute to higher TQ and reduced scalping. Although this appears to be the case in connection with TE applications, this response was only seen in summer 2012 and not 2013. It may be that less mature turf grows more aggressively or was simply more susceptible to mowing frequency relative to more mature kikuyugrass turf. Differences in responses of older and younger kikuyugrass stands to stimuli such as N application have been documented by Barton et al. (2009). Increased turfgrass firmness in both years with higher mowing may have been due to the more frequent use of the Baroness mower, which is relatively heavier compared to other walk-behind mowers. Mowing 6 times wk^{-1} is very labor and resource intensive and cannot be justified by the limited benefits found in this study.

Nitrogen

Higher N fertility on kikuyugrass turf is often associated with increased scalping. For this reason many kikuyugrass managers apply very low N rates to their turf. The lack of significant ratings in 2012 when compared to 2013 could have been due to stand age. Barton et al. (2009) described older stands needing less N to maintain the same quality as younger stands of kikuyugrass. Overall, our data seem to support the commonly used practice of reduced N fertility on this species. Lower N reduces excessive growth which helps to limit scalping. Managers should continue to use low N amounts even on heavily managed kikuyugrass turf.

Fungicides

Fungicide treatments had very little effect on ratings in either year of the study. It is not understood why fungicide applications were associated with firmer kikuyugrass from summer 2012 through spring 2013. The turfgrass pathogen *Rhizoctonia solani* AG2-2 also known as kikuyu large patch, was present in the winter of 2012 and spring of 2013. Fungicide control of this pathogen is what most likely produced the significantly higher TQ and NDVI ratings in spring and summer of 2013.

CONCLUSIONS

Trinexapac-ethyl application every two weeks, verticutting 2 times yr^{-1}, and mowing six times wk^{-1} produced the highest quality kikuyugrass turf. While in some ratings individual treatments did have some negative effects, overall the combination of these treatments contributed positively to kikuyugrass turf quality. Monthly fungicide applications contributed to higher quality kikuyugrass by preventing disease and may help produce firmer turfgrass. Nitrogen fertilization had minor effects. Higher N often improved turf color but also added to scalping. Future research should focus on improvement of kikuyugrass to develop a turf-type cultivar that requires less intensive management.

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Table 2.1. Monthly average air temperature (air temp.; °C), precipitation (precipitation; mm), reference evapotranspiration (ET_{OS}) for the University of California, Riverside Turfgrass Research Facility (Riverside, CA) during the study period.

Climate Parameters	Jan.	Feb.	Mar.	April	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Annual Mean
	°C												
Air temp. 2012	14	12.9	13.3	16.5	19.1	20.7	23.6	26.9	25.6	20.1	15.7	11.3	18.3
Air temp. 2013	11.4	11.8	15.6	16.6	19.8	21.8	24.1	24.4	23.6	17.7	15.6	12.9	17.9
	mm												
Precipitation 2012	9.6	16.2	24.5	22.1	1.1	0	1.9	4.5	0.2	4.4	9.7	40.3	11.2
Precipitation 2013	15.3	21.4	16.8	0.1	6.3	0	8.9	30.4	0	13	30.5	9.9	12.7
ET_{os} 2012	77	87	115	149	178	194	201	199	164	111	69	43	132
ET_{os} 2013	69	81	122	145	178	187	181	187	156	109	70	71	130

Table 2.2. Fungicide treatment regime. The same regime was used for 2012 and 2013.

Fungicides	Jan.	Feb.	Mar.	April	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
Flutolanil 6.4 kg a.i. ha^{-1}											X	
Thiophanate-methyl 3 kg a.i. ha^{-1}						X	X			X		X
Chlorothalonil 8.2 kg a.i. ha^{-1}					X	X	X			X		X
Propiconazole 1 kg a.i. ha^{-1}	X	X	X		X							
Azoxystrobin 0.3 kg a.i. ha^{-1}				X				X	X			

Table 2.3. Results of ANOVA testing the effects of nitrogen, mowing frequency, fungicides, trinexapac-ethyl (TE), and cultivation practice and their interactions on turf quality (TQ), Normalized Difference Vegetation Index (NDVI), scalping, turfgrass firmness, ball roll distance (BRD), and tension of 'Whittet' kikuyugrass for spring 2012 and 2013.

Source	TQ		NDVI		Scalping		Firmness		BRD		Tension	
Year	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013
Nitrogen	NS[†]	NS	NS	**	NS	*	NS	NS	NS	NS	NS	NS
Mowing	NS	**	**	**	NS	**	***	*	**	**	NS	NS
Fungicide	NS	***	NS	*	NS	NS	*	**	NS	NS	**	NS
TE	***	**	***	*	***	***	***	***	***	***	***	*
Cultivation	*	***	***	***	NS	NS	***	***	NS	**	***	NS
Mow X Cult	*	NS	*	NS	*	NS	***	NS	NS	NS	NS	NS
Mow X Fungicide	NS	**	NS	NS	NS	NS	NS	NS	NS	**	NS	NS
Fungicide X Cult	NS	*	NS	NS	NS	**	NS	NS	NS	*	NS	NS
Mow X Fung X Cult	NS	**	NS	***	NS	NS	NS	**	NS	NS	NS	NS
Mow X TE X Cult	NS	NS	*	NS	NS	NS	NS	NS	NS	**	NS	NS
N X TE X Cult	NS	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
N X Fung X Cult	NS	NS	NS	NS	NS	NS	NS	NS	NS	*	NS	*

* Significant at the 0.05 level of probability
** Significant at the 0.01 level of probability
*** Significant at the 0.001 level of probability
[†]NS Not significant at the 0.05 probability level

Table 2.4. Results of ANOVA testing the effects of nitrogen, mowing frequency, fungicides, trinexapac-ethyl (TE), and cultivation practice and their interactions on turf quality (TQ), Normalized Difference Vegetation Index (NDVI), scalping, turfgrass firmness, ball roll distance (BRD), and tension of 'Whittet' kikuyugrass for summer 2012 and 2013.

Source	TQ		NDVI		Scalping		Firmness		BRD		Tension	
Year	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013
Nitrogen	NS[†]	*	*	***	NS	**	NS	***	NS	***	NS	*
Mowing	***	NS	***	*	***	NS	***	***	**	NS	***	NS
Fungicide	NS	*	NS	***	NS	NS	***	NS	NS	NS	NS	NS
TE	***	***	***	***	***	***	***	***	***	*	*	***
Cultivation	***	NS	***	NS	***	***	NS	***	***	***	***	***
Mow X Cult	*	NS	*	NS	NS	NS	**	NS	NS	NS	***	NS
Mow X TE	NS	NS	NS	**	**	NS	NS	NS	NS	NS	NS	NS
Mow X Fungicide	NS	**	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
N X Cult	NS	***	NS	***	NS	***	NS	NS	NS	NS	NS	NS
N X TE	NS	***	NS	NS	NS	***	NS	NS	NS	NS	NS	NS
TE X Cult	NS	***	NS	***	NS	***	NS	NS	NS	NS	NS	NS
Mow X TE X Cult	NS	NS	NS	NS	NS	NS	**	*	***	**	NS	*
N X Mow X Fung	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	***	NS

Mow X Fung X TE	NS	*	NS	NS	NS	*	NS	NS	NS	NS	NS	NS
N X TE X Cult	NS	NS	NS	NS	NS	***	NS	NS	NS	NS	NS	NS

* Signifcant at the 0.05 level of probability
** Signifcant at the 0.01 level of probability
*** Signifcant at the 0.001 level of probability
†NS Not significant at the 0.05 probability level

Table 2.5. Results of ANOVA testing the effects of nitrogen, mowing frequency, fungicides, trinexapac-ethyl (TE), and cultivation practice and their interactions on turf quality (TQ), Normalized Difference Vegetation Index (NDVI), scalping, turfgrass firmness, ball roll distance (BRD), and tension of 'Whittet' kikuyugrass for summer 2012 and 2013.

Source	TQ		NDVI		Scalping		Firmness		BRD		Tension	
Year	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013
Nitrogen	***	***	***	***	**	NS[†]	NS	***	NS	NS	NS	*
Mowing	NS	*	***	NS	NS	NS	NS	***	*	NS	NS	NS
Fungicide	NS	*	NS	NS	NS	NS	***	NS	NS	NS	NS	NS
TE	*	***	NS	***	**	***	***	***	***	***	***	***
Cultivation	NS	***	***	***	***	***	***	***	***	***	***	***
Mow X Cult	NS	NS	NS	*	NS	NS	**	NS	NS	**	NS	NS
Fung X Cult	NS	***	NS	*	NS	NS	NS	NS	NS	NS	NS	NS
Mow X Fungicide	NS	**	NS	NS	NS	NS	NS	NS	NS	NS	*	NS
N X Cult	NS	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
N X TE	NS	NS	NS	*	NS	NS	NS	NS	NS	NS	NS	*
TE X Cult	NS	***	NS	***	NS	***	NS	NS	NS	NS	NS	NS
Mow X Fung X Cult	**	NS	**	NS	**	NS	**	NS	NS	NS	NS	NS
Mow X TE X Cult	NS	NS	NS	NS	NS	NS	*	NS	NS	NS	NS	NS
N X Fung X TE	NS	NS	NS	NS	NS	NS	*	NS	NS	NS	NS	NS
N X Fung X Cult	NS	NS	NS	NS	NS	NS	NS	NS	NS	*	NS	NS

*	Significant at the 0.05 level of probability
**	Significant at the 0.01 level of probability
***	Significant at the 0.001 level of probability
†NS	Not significant at the 0.05 probability level

Table 2.6. Influence of trinexapac-ethyl on 'Whittet' kikuyugrass TQ, NDVI, scalping, firmness, ball roll, and tension for fall 2012 and spring 2013 in Riverside, CA.

	TQ[†]		NDVI[‡]		Scalping	
	Fall 2012	Spring 2013	Fall 2012	Spring 2013	Fall 2012	Spring 2013
TE[§]	Means		Means		Means	
	1-9		0-1		%	
TE1	5.4b[¶]	5.9b	0.73a	0.72b	8.1a	2.1a
TE0	5.6a	6.1a	0.73a	0.73a	5.4b	0.8b
	Firmness[#]		BRD[††]		Tension	
	Means		Means		Means	
	G_{Max}		Meters		kg m^{-1}	
TE1	35.8b	26.5b	1.54a	1.28a	4.1b	4.5b
TE0	37.8a	28.9a	1.49b	1.25b	4.3a	4.6a

[†] Turf quality ratings were measured on a 1–9 scale, with 9 = best turf. TQ < 6 is deemed commercially unacceptable.
[‡] NDVI, Normalized difference vegetation index.
[§] TE, trinexapac-ethyl.
[¶] Within columns, means followed by the same letter are not significantly different according to Fisher's LSD ($\alpha = 0.05$) test.
[#] Clegg Impact Soil Tester measuring firmness in G_{Max}.
[††] BRD, Ball roll distance.

Table 2.7. Influence of weekly mowing frequency, and trinexapac-ethyl treatments on 'Whittet' kikuyugrass scalping for summer 2012 in Riverside, CA.

		Scalping
		2012
Mow	TE[†]	Means
		%
6	TE1	4.9b[‡]
6	TE0	7.1b
3	TE1	8.3b
3	TE0	17.0a

[†] TE, trinexapac-ethyl.

[‡] Within columns, means followed by the same letter are not significantly different according to Fisher's LSD ($\alpha = 0.05$) test

Table 2.8. Influence of weekly mowing frequency, fungicide, and trinexapac-ethyl treatments on 'Whittet' kikuyugrass TQ and scalping for summer 2013 in Riverside, CA.

			TQ[†]	Scalping
			2013	2013
Mow	Fung[‡]	TE[§]	Means	Means
			1-9	%
6	F1	TE1	6.9ab[¶]	0.8d
		TE0	5.9cd	7.8ab
	F0	TE1	6.8ab	0.7d
		TE0	6.1c	5.1c
3	F1	TE1	7.0a	0.1d
		TE0	6.1c	6.5bc
	F0	TE1	6.8b	0.3d
		TE0	5.8d	9.4a

[†] Turf quality ratings were measured on a 1–9 scale, with 9 = best turf. TQ < 6 is deemed commercially unacceptable.
[‡] Fung, fungicide applications every month based preventative fungicide regime.
[§] TE, trinexapac-ethyl.
[¶] Within columns, means followed by the same letter are not significantly different according to Fisher's LSD ($\alpha = 0.05$) test.

Table 2.9. Influence of trinexapac-ethyl, and cultivation treatments on 'Whittet' kikuyugrass Normalized Difference Vegetation Index for summer 2013 in Riverside, CA.

		NDVI[†]
		2013
TE[‡]	Cult[§]	Means
		0-1
TE1	V	0.78b[¶]
	G	0.80a
TE0	V	0.75c
	G	0.73d

[†] NDVI, Normalized difference vegetation index.
[‡] TE, trinexapac-ethyl.
[§] Cultivation treatment; V, heavy verticutting 2 times yr^{-1}; G, grooming 3 times wk^{-1}.
[¶] Within columns, means followed by the same letter are not significantly different according to Fisher's LSD ($\alpha = 0.05$) test.

Table 2.10. Influence of weekly mowing frequency, trinexapac-ethyl, and cultivation treatments on 'Whittet' kikuyugrass firmness, ball roll distance, and tension for summer 2012 and 2013 in Riverside, CA.

			Firmness[†]		BRD[‡]	Tension
			2012	2013	2012	2012
Mow	TE[§]	Cult[¶]	Means	Means	Means	Means
			G_{Max}		Meters	kg m^{-1}
6	TE1	V	31.6a[#]	35.4ab	1.36c	4.49bc
		G	28.1b	24.6f	1.46a	4.06e
	TE0	V	32.3a	37.0a	1.37bc	4.60ab
		G	32.0a	29.1d	1.39bc	4.31cd
3	TE1	V	25.7c	31.3c	1.40b	4.31cd
		G	28.1b	24.1f	1.40b	3.99e
	TE0	V	29.4b	34.8b	1.30d	4.72a
		G	29.1b	26.5e	1.37bc	4.15de

[†] Clegg Impact Soil Tester measuring firmness in G_{Max}.
[‡] BRD, Ball roll distance.
[§] TE, trinexapac-ethyl.
[¶] Cultivation treatment; V, heavy verticutting 2 times yr^{-1}; G, grooming 3 times wk^{-1}
Within columns, means followed by the same letter are not significantly different according to Fisher's LSD ($\alpha = 0.05$) test.

Table 2.11. Influence of weekly mowing frequency, fungicide treatments, and cultivation treatments on 'Whittet' kikuyugrass TQ, NDVI, firmness, and scalping for fall 2012 and spring 2013 in Riverside, CA.

			TQ [†]		NDVI [‡]		Firmness[§]		Scalping
			Fall 2012	Spring 2013	Fall 2012	Spring 2013	Fall 2012	Spring 2013	Fall 2012
Mow	Fung[¶]	Cult[#]	Means	Means	Means	Means	Means	Means	Means
			1-9		0-1		G_{Max}		%
6	Fung1	V	5.4ab[††]	5.9b	0.75ab	0.72de	42.9a	31.9a	5.1cd
		G	5.6a	6.3a	0.73bc	0.74bc	34.2cd	25.5ef	7.7bc
	Fung0	V	5.6a	5.9b	0.76a	0.72e	39.1b	30b	3.1d
		G	5.5ab	6.1ab	0.72c	0.72de	32.1d	25.8de	8.6ab
3	Fung1	V	5.6a	6ab	0.73bc	0.73de	38.7b	29.1bc	4.7cd
		G	5.3b	6.2ab	0.7d	0.74ab	36.1c	37.4cd	11.5a
	Fung0	V	5.4ab	6ab	0.72c	0.71e	39.3b	28.3bc	6.3bc
		G	5.6a	6ab	0.73c	0.75a	32.2d	23.7f	7.0bc

[†] Turf quality ratings were measured on a 1–9 scale, with 9 = best turf. TQ < 6 is deemed commercially unacceptable.
[‡] NDVI, Normalized difference vegetation index.
[§] Clegg Impact Soil Tester measuring firmness in G_{Max}.
[¶] Fung, fungicide applications every month based preventative fungicide regime.
[#] Cultivation treatment; V, heavy verticutting 2 times yr^{-1}; G, grooming 3 times wk^{-1}.
[††] Within columns, means followed by the same letter are not si gnificantly different according to Fisher's LSD ($\alpha = 0.05$) test.

Table 2.12. Influence of nitrogen, and cultivation treatments on 'Whittet' kikuyugrass turf quality and scalping for summer 2013 in Riverside, CA.

		TQ[†]	Scalping
		2013	2013
N[‡]	Cult[§]	Means	Means
kg $ha^{-1}yr^{-1}$		1-9	%
244	V	6.6a[¶]	0.7c
	G	6.4bc	9.1a
98	V	6.3c	1.2c
	G	6.5ab	4.3b

[†] Turf quality ratings were measured on a 1–9 scale, with 9 = best turf. TQ < 6 is deemed commercially unacceptable.
[‡] N, nitrogen rates.
[§] Cultivation treatment; V, heavy verticutting 2 times yr^{-1}; G, grooming 3 times wk^{-1}.
[¶] Within columns, means followed by the same letter are not significantly different according to Fisher's LSD ($\alpha = 0.05$) test.

Table 2.13. Influence of weekly mowing frequency, and cultivation treatments on 'Whittet' kikuyugrass TQ, and NDVI and tension for summer 2012 in Riverside, CA.

		TQ[†]	NDVI[‡]
		2012	
Mow	Cult[§]	Means	Means
		1-9	0-1
6	V	6.7a[¶]	0.81a
	G	5.6b	0.72b
3	V	5.6b	0.74b
	G	5.2c	0.69c

[†] Turf quality ratings were measured on a 1–9 scale, with 9 = best turf. TQ < 6 is deemed commercially unacceptable.
[‡] NDVI, Normalized difference vegetation index.
[§] Cultivation treatment; V, heavy verticutting 2 times yr^{-1}; G, grooming 3 times wk^{-1}.
[¶] Within columns, means followed by the same letter are not significantly different according to Fisher's LSD ($\alpha = 0.05$) test.

Chapter 3

Attempts at Androgenesis through Anther Culture in Kikuyugrass

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Abstract

Depending on its location, kikuyugrass (*Pennisetum clandestinum* Hochst. ex Chiov.) is considered either an invasive weed or a desired species on golf courses and other turf areas along coastal and inland California. Kikuyugrass is a tetraploid species with 2n = 4x = 36 chromosomes. As part of a comprehensive project aimed at kikuyugrass improvement and management, androgenesis was attempted to create a diploid and presumably less aggressive turf-type cultivar. Androgenesis had not been attempted on kikuyugrass previously, thus a protocol used on pearl millet (*Pennisetum glaucum*), a closely related species, was used. Two different culture media were tested: C17 has been successful with cool-season grasses and the Yu-Pei medium was successful on pearl millet. Inflorescences at the uninucleate microspore stage were harvested in the field and surface sterilized using 70% ethanol and 5% bleach (NaClO). Anthers were plated onto culture media in sterile conditions and incubated for 7 d at 14 °C, 7 d at 20 °C, then 4 wk at 26 °C. A total of 4665 anthers were plated on the C17 and Yu-Pei media. No signs of mitosis were seen on any of the observed anthers and no embryos were formed. To check if failure occurred due to the species or the process, the same protocol was performed on 25,335 pearl millet 'Tifgrain 102' anthers. No embryos were formed. Based on these results, the possibility of successful androgenesis in kikuyugrass remains an open question.

Turfgrasses are a common part of the landscape in most urban areas in the United States. They are used for aesthetics, functional purposes (e.g., noise, glare, dust reduction), and for sporting activities. Of particular importance in California is kikuyugrass (*Pennisetum clandestinum* Hochst. ex Chiov.). Originally from the Kenyan Highlands of east Africa it has been introduced into, or invaded, every continent except Antarctica (Mears, 1970). In the early 20th century kikuyugrass was introduced into California for erosion control but soon escaped and invaded other areas throughout the state, especially southern coastal regions. It is a prostrate perennial grass which spreads via seed and vegetatively by rhizomes and stolons. Kikuyugrass has been identified as a noxious weed and invasive species by the U.S. federal government (USDA, 2015). Thus, some turf managers seek to eradicate it using various chemical controls (Cudney, et al. 1993). However, a growing number of venues, namely golf courses and athletic fields, effectively manage this species as a desired playing surface. As a warm-season turf species, kikuyugrass possesses good heat, drought, and salinity tolerance, and exceptional winter color retention (Mears, 1970; Morris, 2009).

Kikuyugrass is a tetraploid with the somatic chromosome number of 2n= 36. It is an outcrossing species that has small bisexual flowers with 2-4 sub spikelets. Although there has been some research on and improvement of kikuyugrass as a forage in other countries, relatively little research has been done on kikuyugrass as a turf species. There are only two cultivars (Whittet and AZ-1), both of which are forage types that are propagated in California. Due to its invasive status kikuyugrass germplasm is very

difficult to import. Thus it is vital to utilize and improve the genetic resources available in California to create a turf-type cultivar.

The major problem with kikuyugrass is its aggressive growth habit. Extensive rhizomes and especially stolons can invade areas where it is not wanted, and its vertical growth habit and heavy thatch production can lead to frequent turf scalping in maintained stands (Gross, 2003). A potential mechanism to reduce the aggressive growth in kikuyugrass is through ploidy reduction.

Ploidy manipulation to develop new traits is common in domesticated plants. As a general rule, higher vigor and larger biomass are expected in higher ploidy levels. Polyploids created through chromosome doubling are sometimes desirable for their increased cell size and enlarged plant organs (Emsweller and Ruttle, 1941; Schepper et al., 2001). Triploidy is routinely used to produce seedless cultivars (Acquaah, 2007). Based on these facts, an assumption was made that ploidy reduction in kikuyugrass would reduce plant vigor, thus make it more suitable for turf, and perhaps also create sterile accessions. Ploidy reduction can be achieved through androgenesis.

Androgenesis is the process of induction and regeneration of haploids and double haploids from microspores. It was first developed in *Datura inoxia* when Guha and Maheshwari (1964) discovered that immature pollen can be induced to switch its normal gametophytic development to sporophytic and produce haploid plants. Generation of haploids through androgenesis has been successful in a very wide range of species, including pearl millet and maize, both of which are closely related to kikuyugrass (Nitsch et al., 1982; Budak et al., 2004). It has also been used in the turfgrass species *Lolium*

perenne and *Festuca pratensis* (Kopecky et al., 2005). Androgenesis is most commonly used to create doubled haploid plants (Sood and Dwivedi, 2015). Chromosome doubling is usually associated with increased plant size. The opposite effect could be expected for haploid production, and has been recorded in pepper, *L. perenne*, and *F. pratensis* (Dolcet-Sanjuan et al., 1997; Kopecky et al., 2005).

This study was conducted to test if androgenesis was feasible in kikuyugrass and if the resulting diploid plants showed reduced vigorous growth. To achieve this, previously tested procedures were attemptedd (Nitsch et al., 1982). The same process was later applied to pearl millet to confirm methodology used to produce embryos.

Materials and Methods

Kikuyugrass Harvesting and Plating I

The standard stage in male gametogenesis susceptible to switch from the gametophytic to sporophytic pathway is the late uninucleate microspore stage. Spikelets were harvested during early development when they were still housed in the sheath prior to anther emergence. Individual anthers were excised from each spikelet and live stained in 1% acetocarmine solution, squashed on microscope slides in a drop of 1% acetocarmine and heated to near boiling. The slides were then viewed under a light microscope. The uninucleate stage is easy to identify: the microspores are round with an obvious pore and a single nucleus. This stage is present when the spikelets emerge from the flag leaf sheath.

For anther culture, inflorescences were harvested daily from a maintained field of 'Whittet' kikuyugrass. The turfgrass field was 27 m x 18 m and was mowed at a height of 1 cm 3 times wk^{-1}. Kikuyugrass rarely flowers unless stressed in some way. Frequent mowing allowed for continuous flowering and anther harvest. In Riverside, California where the study was conducted, kikuyugrass can be induced to flower from April until October and anthers were harvested during this entire period.

Stems with spikelets emerging from the leaf sheath were harvested in the morning one day after mowing. Inflorescences were harvested with a pair of forceps and small scissors, extra tissue removed, spikelets were surface sterilized with 70% ethanol solution for 45 s, 5% bleach (NaClO) solution for 6 min and rinsed three times in sterile water.

Sterilized spikelets were dissected under a dissection microscope in a laminar flow hood and individual anthers were placed onto 10-cm plastic Petri dishes containing C17 media (Table 3.1). All tools were autoclaved for 30 min. For the first 2000 anthers the plates were sealed with parafilm and placed in a dark box at room temperature in the lab. They were checked daily for signs of embryo or callous formation.

Kikuyugrass Harvesting and Plating II

For the second round of plating the methods were those that had been tested in more closely related species, pearl millet and maize (*Zea maize*) (Nitsch el al., 1982). Uninucleate stage discovery, harvesting, sterilization and plating were all done using the same methods described above. Media preparation and incubation parameters were done according to Nitsch et al., (1982). The medium used was modified Yu-Pei medium described in Nitsch et al., (1982) and listed in Table 3.2. After plating, the anthers were

placed in an incubator for 7-10 d at 14 °C and transferred to 20 °C for 7 d, both in the dark. Next they were transferred to 26 °C for 4 weeks under a photoperiod of 16 h day/8 h night. During this process the plates were observed daily for infection, embryo and callus formation. At the end of the 6-wk incubation, a few anthers were collected from plates, stained in 1% acetocarmine and checked for signs of mitosis.

Pearl millet harvesting and plating

To test the method itself, androgenesis was attempted on pearl millet. Pearl millet cv. Massue had been successfully used in androgenesis (Nitsch el al., 1982). Unfortunately, no seed samples of this cultivar could be located in collections. Thus, the grain cv. Tifgrain 102 was used, obtained from the University of Georgia, Tifton. Seeds were germinated on wet soil in trays in the greenhouse at temperatures between 18 °C and 29 °C. After 7-10 d 10 seedlings were selected and transplanted to individual 18 cm x 30 cm plastic pots. Plants were fertilized immediately after transplantation with the complete fertilizer ($16N–16P_2O_5–16K_2O$) at 5 g per pot, then again 2 weeks later.

Six panicles were harvested at different developmental stages to determine the timing of the uninucleate microspore stage. Testing was done in the same fashion as previously. In ‘Tifgrain 102’ the uninucleate stage occurs when panicles have emerged between 30% and 50% from the sheath of the flag leaf.

For androgenesis, millet planting began on 6 Jan 2015 and continued until Aug 2015 with seeds being germinated every 10-14 d. Harvesting occurred circa 40 d after germination. Panicles were cut below their base, placed in plastic bags and taken to the lab. To confirm that each panicle contained microspores at the uninucleate stage, several

spikelets in each panicle were dissected, live stained and screened under a light microscope. Usually, testing 4-5 spikelets along the length of the panicle was sufficient to determine which section contained the uninucleate microspore stage. This section was excised and surface sterilized using the protocol above. Individual florets, consisting of 2 spikelets each, were plated on the growth media. The incubation regime was the same as the second round of kikuyugrass anthers. At the end of the incubation period anthers were sampled and tested for signs of mitosis.

Results

In the first round of plating of kikuyugrass 1,714 anthers were plated on the C17 medium. The infection rate was high with seven out of nine plates developing varying levels of infection before the 6 wk incubation period was finished. Only 334 anthers (19%) made it to the end of the 6-wk incubation period without infection. No anthers on any of the nine plates showed any embryo or callous formation indicating androgenesis had not occurred.

In the second round, a more concerted effort was made to reduce the infection level. A total of 2,951 anthers were plated on 14 plates and only three plates with 912 anthers were infected. A total of 2,039 (69%) made it to the end of the 6-wk incubation period without infection but there were no signs of embryogenesis or callus formation. Microscopic screening did not detect any signs of mitosis in microspores.

Pearl millet anatomy and the ability to directly plate florets rather than individual anthers allowed for a more efficient and streamlined plating process. A total of 25,335

anthers were plated on 31 plates. Six plates with 5,516 anthers were lost to infection. The remaining 19,809 (78%) anthers made it to the end of the 6 wk incubation period. A subset (8,175) of the total 25,335 pearl millet anthers was kept in the dark for the entire incubation period. No embryo or callous formation was observed on any anthers and no mitosis was observed in anthers tested during or after the 6-wk incubation period.

Discussion

Based on the results of the three androgenesis attempts, it is still not clear if androgenesis is a viable approach to ploidy reduction in kikuyugrass. No mitosis was observed in microspores in any of the three attempts, either kikuyugrass or pearl millet, and no embryoids or callus were formed. The data are inconclusive. While no androgenesis has ever been done in kikuyugrass, pearl millet microspores have been successfully cultured on artificial media. However, in most cases androgenesis is genotype-specific, with very wide variation in androgenetic response among different lines/accessions of a species. This has been observed in wheat, citrus, rice, and grapevines (Jones and Petolino, 1987; Sood and Dwivedi, 2015; Chen, 1985; Guha-Mukherjee, 1973; Gresshoff and Doy 1974). It is unfortunate that in this study the cultivar of pearl millet known to be amenable to androgenesis could not be located. The cultivar used instead does not in any way prove whether the method was ineffective or the genotype was non-responsive.

The first attempt at androgenesis was done using effective and proven methods for cool-season grasses (C3). Perhaps this approach is unsuitable for a warm-season

grass such as kikuyugrass. Moreover, in almost all cases of successful androgenesis some form of stress must be applied to collected stems, before anther plating (Sood and Dwivedi, 2015). This stress makes the switch from the gametophytic to sporophytic pathway of development possible. Very often, this stress is applied in the form of cold treatment for up to several weeks. With a warm season species such cold treatment was not feasible and so no stress was applied at all. And lastly, high infection rate reduced the size of experiment to a very small one indeed.

Based on the experience from the first attempt it was expected that the second attempt would yield viable embryos from kikuyugrass microspores. The protocols for this attempt were based on the successful androgenesis of pearl millet and maize. Pearl millet is within the same genus as kikuyugrass (*Pennisetum*) and maize is also closely related (Budak et al., 2004). The same medium, temperature regimes, and incubation times were used on kikuyugrass as was successfully used on these species (Nitsch et al., 1982). It was expected that if a protocol was successful on a closely-related species it might also work for kikuyugrass. Unfortunately, no successful androgenesis in kikuyugrass was observed again.

To determine the role that methodology might have played in unsuccessful attempts, the same protocol was used on pearl millet itself, cv. Tifgrain 102. Even this was unsuccessful. The failure could be due to multiple factors. It may be that the genotypes 'Whittet' kikuyugrass and 'Tifgrain 102' pearl millet are genetically unsuited for androgenesis and that successful embryos cannot be formed in these accessions regardless of the protocol. While androgenesis has been successful on pearl millet, cv.

‘Tifgrain 102’ has never been tested. Genotype specific androgenesis could be linked to the number of microspores within the anther and their robustness, the nutrients present in the anther tissues, the endogenous levels of plant growth regulators or a combination of these effects (Sood and Dwivedi, 2015).

It cannot be entirely discounted that the entire species of kikuyugrass is unsuited for androgenesis. This has been seen in onions, leeks, and many perennial and woody species (Keller, 1990; Sood and Dwivedi, 2015). In pearl millet, results have been mixed with some researchers having success in all cultivars tested (Bui Dang Ha and Pernes, 1982; Nitsch et al., 1982; Panchangam and Mallikarjuna, 2014) while others had no success with any (Nallni and Bhalla, 1987). Other than Nitsch et al., (1982) the cv. Massue has not been tried again in any androgenesis experiments, and androgenesis has never been attempted with ‘Tifgrain 102’ before this study.

Growing conditions of the donor plants are also known to affect androgenesis success. Differences in competence of the cultured microspores for embryogenic development have been observed from field-grown and pot-grown plants (Datta 2005; Silva 2010). Similar variability has been noted in plants grown in different seasons (Foroughi-Wehr and Mix 1979). At times, infected, diseased, and unhealthy plants have lower androgenic response; in some other cases any stress, including biotic or abiotic, appears to promote the switch to sporophytic microspore development. Kikuyugrass in this study were grown in a healthy setting with no disease pressure. However, the research plot was maintained under golf course fairway conditions which may have exposed the grass to stress via weekly mowing. This was unavoidable since mowing is

required to induce flowering. The research plot was not subject to any water or nutrient stresses over the course of the project. Overall, inflorescences were produced under near ideal conditions and it seems unlikely that they failed due to poor health.

It cannot be entirely discounted that inexperience was a contributing factor. While all steps were taken according to published protocols, minor deviations might have had an impact. It is a common observation in almost any protocol that early attempts are less successful and the efficiency improves with time and repetition. The failure in both species makes pinpointing a direct cause difficult. However, any future attempts at androgenesis in kikuyugrass should focus on three aspects: larger scale, multiple genotypes and different conditions. A much larger project could be set up where multiple kikuyugrass genotypes are tested simultaneously with multiple protocols. This was done successfully in pearl millet and allowed researchers to choose the best combination for future double haploid production (Panchangam and Mallikarjuna, 2014).

Suspension culture of isolated microspores rather than anther culture may be more effective. At the very least it would dramatically increase sample sizes and hence, the probability of success. Microspore culture uses crushing or blending to free the microspores from the inflorescence. The resulting slurry is filtered and centrifuged to isolate the microspores. After filtering the microspores are suspended in medium until embryos or callous tissue is formed.

Diploid kikuyugrass may also be generated via gynogenesis. Gynogenesis produces embryos that are derived from egg cells in plant inflorescences. Gynogenesis has a serious shortcoming in its low throughput, in addition to the same hurdles that

plague androgenesis (genotype specificity, media selection). Despite this, it has been attempted in barley, rice, wheat, maize, and pearl millet (Thammiraju, 2000; Keller and Korzun, 1996) and should also be attempted in kikuyugrass.

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Table 3.1. Composition of C17 basal medium at pH 5.8 (1N NaOH).

C17 Medium	Amount
Components	mg/L
KNO_3	1400
NH_4NO_3	300
KH_2PO_4	400
$MnSO_4 \cdot 4H_2O$	11.2
$CaCl_2 \cdot 2H_2O$	150
$MgSO_4 \cdot 7H_2O$	150
KJ	0.86
$CoCl_2 \cdot 6H_2O$	0.025
H_3BO_3	6.2
$ZnSO_4 \cdot 7H_2O$	8.6
H_3BO_3	1.6
$CuSO_4 \cdot 5H_2O$	0.025
$FeSO_4 \cdot 7H_2O$	27.8
$Na_2EDTA \cdot 2H_2O$	10^{-4}M
Pyridoxine HCl	0.5
Nicotinic Acid	0.5
Thiamin HCl	1.0
Myo-Inositol	100
Glycine	2.0
Biotin	1.5
Folic Acid	0.5
Maltose	90000
2,4-D	2.0

Table 3.2. Composition of Yu-Pei basal medium at pH 5.8 (1N NaOH).

Yu-Pei Medium	Amount
Components	mg/L
$MnSO_4 \cdot 7H_2O$	4.4
$ZnSO_4 \cdot 7H_2O$	1.5
H_3BO_3	1.6
KI	0.8
FeEDTA	10^{-4}M
Glycine	2.0
Nicotinic Acid	0.5
Thiamin HCl	1.0
Pyridoxine HCl	0.5
TIBA	0.1
Casein hydrolysate	500
1-proline	100
sucrose	60000
Activated charcoal	5000
agarose	6000
KNO_3	2500
NH_4NO_3	165
$CaCl_2 \cdot 2H_2O$	176
KH_2PO_4	510
$MgSO_4 \cdot 7H_2O$	370

Chapter 4

Genetic Diversity of Kikuyugrass Detected Using DArTseq Markers

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Abbreviations: amplified fragment length polymorphism, AFLP; Australia, AUS; California, CA; Diversity Arrays Technology sequencing, DArTseq; DNA amplified fingerprinting, DAF; random amplified polymorphic DNA, RAPD.

Keywords: California, Hawaii, Australia, AWclust, GenAlEx

Abstract

Kikuyugrass (*Pennisetum clandestinum* Hochst. Ex Chiov.) is a warm-season species introduced into California in the 1920s. It is considered a weedy invasive species due to its aggressive growth habit and it is very difficult to eradicate. Perhaps for this reason it is managed on golf courses, athletic fields, and lawns but there is no active breeding in the US. To assess the extent of genetic variation present among available accessions of kikuyugrass local populations were sampled from throughout California, as well as from the collections at the University of California, Riverside (UCR), Hawaii and Australia. A total of 20,000 single nucleotide polymorphism (SNP) makers were discovered using the Diversity Arrays Technology sequencing (DArTseq) platform. The hierarchical plot, gap statistics, and the principal coordinate analysis all showed that the 336 accessions in the study separated into three main clusters. Seventy-seven percent of the total genetic variation was due to within population variation, while 23% represented among population variation. The main axis of the principal coordinate analysis accounted for 32.7% of the total variation. Accessions from California showed the least genetic variation with all but six located in the same cluster. Accessions from Australia and Hawaii showed a much broader degree of genetic diversity and would be valuable stock for breeding should such effort become feasible and the exchange of germplasm possible. Meanwhile, breeding toward new turf-type cultivars must be limited to available germplasm within the UCR collection with select accessions from around the state.

Kikuyugrass is a warm-season turfgrass originating from the east African highlands. The species is prolific worldwide, having been introduced into, or invaded, every continent except Antarctica (Cook et al., 2005). In some countries, including South Africa and Australia, kikuyugrass is widely used as a forage species. Kikuyugrass was introduced into California (CA) in the 1920s for soil stabilization, but quickly spread throughout the state and now inhabits mainly coastal as well as southern and central inland regions (Garner, 1925).

Kikuyugrass has colonized many sports and residential turf areas in CA since its introduction. It has high phenotypic plasticity, thus its morphology and functionality are related to environment, especially cultural management inputs. Low N fertilization, frequent low mowing, use of plant growth regulators, and verticutting help to produce turf of acceptable quality and ca. 100 golf courses in California manage kikuyugrass (Mock et al., 2016; in preparation). Kikuyugrass has some desirable characteristics such as superior winter color retention relative to other warm-season turfgrasses. On the other hand, little or no maintenance with the possible exception of high N tends to result in less desirable turf quality characteristics and invasiveness. Aside from kikuyugrass originally introduced into CA, the most commonly used cultivars in CA is 'Whittet', a forage-type that was developed in Australia in the 1970s and now produced both as seed and sod. Although other cultivars have been developed in Australia, they are either forage types unsuitable for turf, or cultivars that have been discontinued due to underperformance when compared to common kikuyugrass. Overall, it is difficult to distinguish among

different kikuyugrass genotypes even in residential areas compared to intensively managed sports turf like golf course fairways (Morris, 2009).

If kikuyugrass is to become a more broadly utilized turf species, new cultivars must be produced with fewer management inputs and improved quality and pest tolerance. Any breeding progress requires genetic variation. Kikuyugrass is a relatively new introduction in the US and the exact pedigree of various sources/populations is unknown. For all practical purposes and given the aggressiveness of the species, all existing kikuyugrass may have originated from a single introduction and differs only by recent mutations. Hence, before any serious breeding is undertaken, genetic diversity within the available and accessible kikuyugrass resources needs to be studied to ascertain that sufficient variation exists to breed a new turf-type cultivar.

An additional complication to any breeding effort is the fact that kikuyugrass is listed on the federal noxious weed list (USDA, 2014) and any movement of germplasm is restricted. Therefore, understanding the genetic makeup of kikuyugrass in CA is especially important. So far only one genetic variation study has been conducted on kikuyugrass in California. Using isozymes Wilen et al. (1995) found very little genetic diversity among subpopulations collected at three different golf courses representing northern, central, and southern California. A study in Australia used DNA amplified fingerprinting (DAF) and 40 polymorphic loci and identified two main groups among 13 genotypes tested (Holten et al., 2007). Morris (2009), using 195 polymorphic randomly amplified polymorphic DNA (RAPD) found three main groups among 19 genotypes tested and concluded that significant variation did exist within Australian genotypes.

There are two potential sources of genetic variation available in CA. Some variation may exist among accessions throughout the state, either because they represent different introductions or because of different accumulation of mutations. Genetic variation may also be present among accessions maintained at the University of California, Riverside (UCR). These accessions were selected from a bag of kikuyugrass seed imported from Australia with a total of 20 accessions established by Dr. Ruth Shaw between 1990 and1992 (Cockerham et al., 1992). Selection was based on winter color retention, growth habit, sward density, leaf texture, and spread. Cockerham et al. (1992) described significant differences among these accessions. After their initial planting and rating, no further research was conducted on kikuyugrass at UCR for about 20 years and the original plots received only minimal maintenance, such as occasional mowing, fertilization and edging to maintain plot borders. These plots were included in this study to determine if genetic variation still existed among the accessions and to compare with accessions collected throughout California.

Molecular characterization of germplasm collections is important in understanding the variability of genetic resources. Several different marker systems have been used for this purpose, in a range of turfgrass species. Amplified fragment length polymorphisms (AFLP) have been used in the warm-season grasses such as seashore paspalum *(Paspalum vaginatum* Swartz), bermudagrass [*Cynodon dactylon* (L.) Pers.], and zoysiagrass *[Zoysia japonica* Steud, *Zoysia rnatrella* (L.) Merr., and *Zoysia tenuifolia* Auct.] (Zhenbang et al., 2009). AFLP markers were also used to investigate genetic variability among breeder stock and sodded varieties of St. Augustinegrass

[*Stenotaphrum secundatum* (Walt.) Kuntze] (Kimball et al., 2012). Isozyme analysis, DNA amplified fingerprinting (DAF), and random amplified polymorphic DNA (RAPD) markers have all been used to determine genetic diversity in kikuyugrass (Wilen et al., 1995; Holton et al., 2007; Morris, 2009).

The purpose of this study was to determine if genetic variation existed within the CA kikuyugrass accessions, including those collected from around the state and from the UCR collection originating from the Australian germplasm. For comparison, collections in Hawaii and Australia were also sampled. The study is based on single nucleotide polymorphism (SNP) markers via the Diversity Array Technology sequencing (DArTseq) platform. The DArTseq is a relatively new platform: the next generation sequencing (NGS) combined with the existing DArT marker platform is a powerful method for the discovery of single nucleotide polymorphism (SNP) (Sansaloni et al., 2011; Kilian et al., 2012; Courtois et al., 2013; Cruz et al., 2013; Raman et al., 2014). DArTseq uses restriction enzymes to separate low copy sequences from the repetitive fraction of the genome and represents a new implementation of complexity-reduced representations (Altschuler et al., 2000). The main advantage of the DArTseq system is its ability for SNP discovery in species with no previous DNA sequence information. It is also capable of generating thousands of markers for genetic diversity analysis as compared to the hundreds developed with the earlier methods. It has already been used to successfully assess genetic diversity in Lesquerella (*Physaria sp*.) (Cruz et al., 2013).

Materials and Methods

Plant material

A collection of kikuyugrass from within California was assembled at UCR. It included the 20 accessions maintained at UCR since their creation by Cockerham et al. (1992) from seed imported from Australia. The accessions have been maintained in 1.8 × 1.2 m plots with 1.2 m alleyways, in three replications, for the last 24 years. A total of five samples were taken from each accession, two each from the first and second replicate and one from the third replicate. Each sample was labeled by plot and replication and planted in a 10-cm^2 pot in the greenhouse.

To sample the germplasm available in the state a questionnaire was sent to turfgrass managers and others in the turfgrass industry to identify useful collection locations. Managers were asked about potentially useful turf-type traits such as winter color retention, male sterility, and disease and drought tolerance. Based on the answers 96 samples were collected throughout the state, of which 69 were from golf courses and the remainder from parks, beaches, cemeteries, and residential areas. Collection took place between June and November 2013. All samples were collected as single stolons so that only one genotype was present in each sample. These samples were maintained in 10 cm^2 pots in a greenhouse at UCR.

A total of 60 samples were collected from Hawaii. Forty-eight came from the collection originally created by Dr. Ukio Urata at the University of Hawaii, Agricultural Experiment Station (Fukumoto and Lee, 2003). The original site of the collection remains at the Mealani Research Station although individual plots were difficult to

recognize. Selection of individual plants was done by attempting to identify and sample the original plots based on field location and the original plot map. All 48 samples from the Mealani Research Station were dried in situ for shipment to CA and potted in the Mealani Research Station. The potted copies are being maintained in case some samples are discovered to have high or unusual genetic variability. The remaining 12 samples were collected along roadsides, in residential areas, on a golf course, and in pasture on the island of Hawaii. Time and circumstances did not allow for duplicates to be planted and maintained for these 12 samples. The 60 samples for shipment to California were desiccated over silica gel.

All California samples were maintained in pots in a greenhouse at the UCR Agricultural Experiment Station. Plants are cut weekly in summer and biweekly in winter to 5 cm height to prevent hybridization through flowering and rhizomes and especially stolons from invading other pots. Plants were watered 3 times per week in order to maintain adequate soil moisture for plant growth and development. A complete fertilizer (16N–16P–16K) was applied every other month for a yearly total rate of 122 kg N ha^{-1}.

Tissue Harvest and DNA analysis

Approximately 100 mg of fresh leaf tissue was collected from each accession, dried over silica gel under vacuum for three days, and pulverized with steel pellets and sand in a FastPrep-24 instrument (MP Biomedicals, LLC, Santa Ana, CA, USA). DNA extraction was performed according to the protocol listed at the Diversity Array

Technology Ltd website (http://www.diversityarrays.com). The end product was total genomic DNA suspended in TE buffer and stored at -80 °C.

DNA was quantified by a NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and its quality was evaluated on 0.8% agarose gel via electrophoresis. Samples of acceptable quality were diluted to 50–100 ng/µl, loaded onto three 96-well microtiter plates and sent to Diversity Arrays Technology, Pty, Ltd Bruce, ACT, Australia.

The 94 samples originating from the collection at the University of Sydney's Plant Breeding Institute were handled differently. Fresh young leaf tissue was collected into 15 x 10 cm plastic bags each with a 7g desiccation packet, and sent directly to DArT Ltd in Bruce, ACT, Australia. DNA extraction, quantification, and plating were performed as part of a paid service.

DArTseq platform development

Restriction enzymes *Pst*I and *Mse*I were selected for the genomic complexity reduction. DNA samples were processed in digestion/ligation reactions as described in Kilian et al. (2012), but replacing a single *Pst*I-compatible adapter with two different adapters corresponding to two different restriction enzyme (RE) overhangs. The *Pst*I-compatible adapter was designed to include Illumina flowcell attachment sequence, sequencing primer sequence and "staggered", varying length barcode region, similar to the sequence reported by Elshire et al. (2011). The reverse adapter contained the flowcell attachment region and *Mse*I compatible overhang sequence. Only "mixed fragments" (*Pst*I–*Hpa*II) were effectively amplified in 30 rounds of PCR using the following reaction

conditions: 1 min at 94 °C for initial denaturation; 30 cycles each consisting of 20 s at 94 °C for denaturation, 30 s at 58 °C for annealing and 45 s at 72 °C for extension; and finally a 7 min extension step at 72 °C. After PCR, equimolar amounts of amplification products from each sample of the 96-well microtiter plate were bulked and applied to c-Bot (Illumina) bridge PCR followed by sequencing on Illumina Hiseq 2500. The sequencing (single read) was run for 77 cycles. Sequences generated from each lane were processed using proprietary DArT analytical pipelines. In the primary pipeline, the FASTQ files were first processed to filter poor-quality sequences, applying more stringent selection criteria to the barcode region compared to the rest of the sequence. Approximately 2,500,000 (±7 %) sequences per barcode/sample were used in marker calling.

After producing various QC statistics the sequences were aligned against the reference created from the tags identified in the sequence reads generated from all samples. In addition, the short sequence tags were aligned against *Zea Maize*, *Setaria italic* (foxtail millet), and *Sorghum bicolor*, all of which are in the same grass sub-family *Panicoideae* as *Pennisetum* (Budak, 2006). The output files from alignment generated using Bowtie software were processed using an analytical pipeline developed by DArT P/L to produce‘‘DArT score’’ tables and ‘‘SNP’’ tables (Langmead et al., 2009).

Data Analysis

The non-parametric program “Allele sharing distance and Ward's minimum variance hierarchical clustering” (AWclust) was chosen to determine the population structure and genetic variation of the SNPs discovered with DArTseq. AWclust was

chosen in part because of its ease of use and its capability of quickly analyzing large SNP datasets. The SNP data set including all accessions in the study was converted into a text file and loaded into the AWclust software. The software generates a matrix of pair-wise allele sharing distances (ASD).

The distance between individuals *i* and *j* was defined as:

$$D_{ij} = \frac{1}{L}\sum_{l=1}^{L} d_{ij(l)}$$

Where $d_{ij(l)}$ = 0, 1, or 2 if individuals *i* and *j* have two, one, or no alleles in common at the *l* th locus respectively.

The ASD is used by AWclust to perform hierarchical clustering. Elements in the ASD matrix that are close will tend to cluster together (Borg and Groenen. 1997). Hierarchical plots were produced for the total set of 336 accessions and separately for the UCR germplasm collection.

The AWclust uses Ward's minimum variance algorithm to calculate hierarchical plots. The Ward's minimum variance algorithm uses the ASD matrix to cluster individuals. The process starts with each cluster containing a single individual. In each of the succeeding steps the algorithm merges the two groups that will result in the smallest increase in the value of within-cluster variance (Ward, 1963). The pair is then joined and the number of clusters reduced by one. This process continues until one cluster contains all individuals. The cluster variance increases nonlinearly during the cluster building process. This allows a clear indication of where groups separate from each other (Ward and Hook, 1963).

Lastly, AWclust calculates the gap statistic which is useful in impartially verifying the number of clusters in a population. A cluster is represented as *K*, such that $\log(W_k)$, where W_k is the pooled within-cluster sum of squares, is farthest below its null reference distribution curve. This is defined as: $\text{Gap}(k) = E[\log(W_k)] - \log(W_k)$, where E() denotes the expectation from the null reference distribution. In this case, the null reference distribution is a uniform distribution (Tibshirani et al. 2001; Gao and Starmer, 2007).

The same data used in the AWclust were also used in GenAlEx v.6.5 (Peakall and Smouse, 2006, 2012) to create a genetic distance matrix (GDM). When calculated across multiple loci for a given set of samples, the GDM calculated by GenAlEx is equivalent to the tally of state differences among the set of DNA profiles. The GDM was used for the subsequent Principal Coordinates Analysis (PCoA) and Analysis of Molecular Variance (AMOVA). The AMOVA was used to calculate PhiPT which is a measure of population genetic differentiation that is analogous to F_{st}. In order to calculate a significant p value for AMOVA, the number of permutations was set to 999, which is the standard for published data.

Results

The DArTseq platform identified a total of 20,363 markers. The average genotype call rate was 94.5% with scoring reproducibility of 99%. The 20,363 markers were pared down to 16,000 based on the call rate and removal of non-informative markers. The call rate is used to sort makers based on absent data. These curated SNPs

were used in both AWclust and GenAlEx to determine genetic diversity among all 336 accessions. A hierarchical plot was first produced for all 336 accessions together.

The hierarchical plot shows three clusters (*K*). The gap statistic was used to objectively verify *K*. The log (W_k) estimations and gap curve are plotted in the left and right of Fig. 4.1. The gap curve is plotted in the format of Gap (*K*) ± standard deviation of log (W_k). The optimal *K* is the elbow point in the observed log (W_k) plot, which corresponds to the maximizing point in the gap curve. From the plots, the estimated optimal *K* is 3. Multiple runs produced similar results.

The PCoA also shows three clusters in the complete set of data (Fig. 4.2). The main axis through the PCoA matrix accounts for 32.7% of the total variation. Axes two and three accounted for 3.4% and 2.1% respectively. There are accessions from each of the germplasm collections mixed throughout all three clusters. The accessions from Australia (AUS) seem to be the most diverse as they are present in, and more equally dispersed among, each of the three clusters. An AMOVA of the complete data set (Table 4.1) shows a much greater proportion of variation within groups (77%) than among groups (23%). The PhiPT score for the AMOVA was 0.234 at p=0.001.

The hierarchical plot for all 336 accessions is broken up into 3 figures due to its large size. The clusters are referred to as cluster 1, 2, and 3 (CL 1, CL 2, CL 3) and correspond to Figs. 4.3, 4.4, and 4.5 respectively. The main genetic difference among the clusters can be seen between CL1 and CL 2 and CL 3 together. A smaller branch length exists between CL 2 and CL 3. This genetic dissimilarity can be visualized further by

looking at the clusters in Figure 4.2. The furthest cluster on the far right of Figure 4.2 is CL 1, while the middle and left clusters are CL 2 and CL 3 respectively.

The clusters CL 2 and CL 3 in the hierarchical plot do not break down into mostly homogenous groups as they do in CL 1. The only pattern that appears is 30 accessions from HI that create a homogenous sub-cluster of CL 3. CL 2 and CL 3 combined breakdown: 99 UCR (53%), 48 HI (25%), 35 AUS (19%), 6 CA (3%). The accessions created and maintained at UCR are only present in CL 2 and CL 3 of the hierarchical plot, with no overlap of the main CA cluster (in CL 1). This can be seen in Figure 4.2 as well.

Clustering of California accessions

Almost all CA accessions collected throughout the state are located in CL 1 (Figure 4.3). This cluster separates into three smaller sub-clusters. The largest of these sub-clusters has 77 accessions and is 94% CA accessions with only one Hawaii (HI) and four AUS accessions. This one CA sub-cluster contains 84% of all CA accessions. The second largest sub-cluster's consists predominantly of AUS accessions: 45 AUS (75%), 9 HI (15%), 6 CA (10%). The last small sub-cluster is again predominantly AUS accessions with 10 AUS, 2 HI, and 2 CA accessions.

Only six accessions from CA are located in CL 2 and CL 3. Two of them, CA50 and CA51, are most likely 'Whittet' since they all share the same clade. Two more fall close to 'AZ-1' although not close enough to be the same accession (CA35 and CA39) (Figure 4.5). CA35 and CA39 were collected in the golf course rough at Riviera Country Club and Bel-Air Country Club, respectively, in Los Angeles. Riviera Country Club has

had kikuyugrass fairways and rough for the past 80 years. Bel-Air Country Club has a mixture of turfgrasses with kikuyugrass invading some areas in the rough. The two courses are within a few kilometers of each other. However, other samples were taken from both golf courses with no distinctive accessions present. The last two distinctive CA accessions are from the fairway at Mesa Verde Country Club located in Costa Mesa, CA (CA55) and the rough at Mission Viejo Country Club (CA66) (Figure 4.4). Other accessions were also collected from both locations but clustered with the majority of CA accessions in CL 1.

Australian accession clustering

The AUS accessions displayed more variation than those from CA (designated PM in Figures 4.3-4.5). Accessions PM1-19, PM88, and PM89 originated from a larger group of 75 selected from a field of common kikuyugrass that had been left without fertilization or water from 2002 to 2005. All of these accessions cluster together in CL 1 of the hierarchical plot (Figure 4.3). Another set of accessions in the AUS collection are from the naturalized lines from the Camden district (Central Sub-Coastal zone of New South Wales) and are numbered PM26, PM38, PM41, PM45 (dry-land salt tolerant), PM52, PM63, PM86, PM87. All of these accessions are in CL 1 except for PM45 which is in CL 2.

There were 27 AUS accessions from the state of New South Wales (NSW): PM61, PM83, PM84, PM20, PM23, PM25, PM28, PM30, PM31, PM37, PM39, PM42, PM43, PM44, PM46, PM60, PM21, PM27, PM32, PM34, PM40, PM54, PM 58, PM59,

PM80, PM81, and PM85. All of which are located in CL 1 except for PM83, PM20, and PM30 which are in CL 2.

There were seven accessions from other states in AUS that were included in the study; Queensland: PM53 and PM56 located in CL 3 and Cl 1 respectively; Victoria: PM62 located in CL 1; Tasmania: PM57 located in CL 1; South Australia: PM29 and PM55 located in Cl 2 and Cl 1 respectively; Western Australia: PM82 located in the CL 3. These seven accessions show a range of variability across multiple states in AUS.

In AUS seed of some of the commercially available cultivars was sown and in all cases found to yield highly variable populations. The most promising seedlings of each cultivar were selected and two accessions of each variety with promising turf phenotypes were selected and propagated vegetatively. Four accessions from these registered cultivar selections were also included in the AUS germplasm. One selection was taken from 'Breakwell' (PM47) and is located in CL 3. Two selections from 'Noonan', PM71 and PM90, are located in CL 2 and CL 3 respectively with one (PM90) sharing a clade with PM47. The selection from 'Whittet' (PM64) is in CL 3. Three of the four accessions clustered closely together with PM64 located right next to the 'Whittet' and 'AZ-1' accessions collected from CA.

There are four accessions in the AUS germplasm that are considered to represent the original introduction from Africa to AUS in 1918 and 1920. All four accessions (PM28, PM60, PM86, and PM87) locate in CL 1. Another three accessions came from material donated by the NSW Department of Agriculture in 2003 as the best lines from their discontinued program of chemical mutagenesis of 'Whittet'. Two locate in CL 2

(PM49, and PM50) and one in CL 1 (PM51). The last 20 accessions from the AUS germplasm collection are hybrid lines with the pollen parents being either PM49, PM50, or PM51. They are listed with both parents in Table 4.2. Not all parents of hybrid lines with a KC number were included in this study. All 20 hybrids except for PM76 and PM66 locate in CL 2 and most are in the same sub-cluster. The parents of both PM76 and PM66 are located in the same sub-cluster of CL 1 as their progeny.

Hawaiian accession clustering

As listed above, the Hawaiian kikuyugrass germplasm was collected in two separate groups. One group represents a majority of the Hawaiian accessions and originates from the neglected and unmaintained collection created by Dr. Urata at the University of Hawaii in the 1980s. The other, smaller, group was collected around the island of Hawaii in June 2014. Accessions from the Urata germplasm collection are scattered throughout all three major clusters while those collected around the island of Hawaii are present only in CL 1 and CL 2. A large sub-cluster of CL 3 contains 30 accessions all of which come from the Urata collection. Based on records kept by Dr. Urata most accessions from the plots in Hawaii for this project were originally collected in the state of Hawaii or were hybrids of Hawaiian accessions (45). Three were hybrids between Hawaiian accessions and kikuyugrass brought from California (HIB4, HIB1, and HIB9). These three locate in the Hawaiian sub-cluster CL 3 (Figure 4.5). The other 18 Urata collection accessions were spread out between CL 2 (10) and CL 1 (8).

Twelve accessions collected from around the island of Hawaii locate in CL 2 (8) and CL 1 (4). Two from CL 1 (HW4 and HW10) were collected along freeway 11 outside of Kona, HI. The accession HW 8 came from the Waimea-Kohala Airport and is located in the CA sub-cluster in CL 1. HW5 came from a pasture at the Mealani Experiment Station in Kamuela, HI. Three other accessions were collected from the same large pasture at the Mealani Experiment Station but cluster close together in CL 2. Two accessions (HW1 and HW6) were collected at the closed Waimea Golf Course and are also in CL 2. Two cultivars, Hosaka and Waimea, both collected at the Mealani Experiment Station are in the same clade in CL 2 and do not appear to be distinct from one another.

University of California, Riverside accessions

The lines maintained at UCR were studied individually in addition to their relationships with the rest of the accessions in this study. Five samples were taken from each of the three replicated plots. This was done to verify if indeed the same accessions are present on replicated plots (are genetically identical). The results show that no accession has remained pure across all three replicates (Figure 4.6). Only 19 of the 40 sampled plots contained identical samples. These were accessions K36, K171, K92, K261, K163, K257, K14, K294, and C17 in replicate 1. In replicate 2 the accessions with two identical samples were: K261, C11, K171, K257, C17, K163, K76, K154, K294, and K289. Accessions with identical samples in both replicates: K261, K171, K257, C17, K163, K294. The accessions with no identical samples in any replicate included K225, K130, C1, K19, K218, and K50.

Three accessions separate into their own clades with plots A/B and C/D separating further into their own subclades. Two of these accessions (C17A-D and K163A-D) have different branch lengths between the two plots, while one (K171A-D) appears to have very little or no difference in branch length. These three grouping are unique because they show no invasion but still differentiate by plot.

While some of the plots of UCR accessions seem to have remained homogeneous, significant mixing has occurred among the plots. Table 4.3 shows physical distances between mixed plots. Mixing occurred both at short and long distances, with one between plots K19A/C and K14E occurring at 18.1 m.

Discussion

Based on the hierarchical plot including all accessions, the gap statistics, the PCoA, and the AMOVA, it is clear that genetic variation does exists among all four germplasm sources. It is also apparent that all 336 accessions form three main clusters. These are seen in both the hierarchical plot and the PCoA, and are identified in the gap statistic plot.

The presence of genetic variation among the accessions tested is illustrated by several parameters. The first is the large branch length between CL1 and CL 2/CL 3 (Figures 4.3-4.5). Although there appears to be low diversity among accessions within clusters, differences among three clusters are substantial. The second measure of genetic variation is the AMOVA PhiPT score and it is significant. Wright (1978) proposed that a PhiPT score from 0.25 to 0.5 indicated a large differentiation between populations and

the range 0.15 to 0.25 indicated moderate differentiation. The interpretation of PhiPT is more complex than simple reference to this quantitative guide as described by Hedrick (1999). Therefore, in modern population genetic procedures a more important question is whether significant genetic differentiation (PhiPT > 0) is detected or not, and whether this differentiation is biologically meaningful. The AMOVA PhiPT score of 0.234 at p=0.001 in this study indicates that there is significant genetic differentiation among the accessions tested. The percentage of variation shown by axis 1 (32.7%) in the PCoA and the obvious three clusters along that axis give further credence to the argument that substantial variation is present in the tested material.

While there is general clustering of accessions based on geographic location, each group of accessions collected at specific locations has some representatives in other clusters. Most importantly all germplasm collections had accessions in CL1 and either CL 2 or CL 3. Only the UCR accessions had limited overall diversity with accessions only in CL 2 and CL 3. This dispersal across clusters was why the AMOVA showed that 74% of the total variation came from within the populations.

The California collection at UCR and samples collected from around the state as a whole showed the least genetic variation. All but six CA accessions were grouped in cluster CL 1. This confirms the findings by Wilen et al., (1995) who found little genetic diversity among samples from three golf courses in CA. Of the six accessions in other clusters only four can be considered distinct since it is highly likely that two were taken from a golf course that had planted 'Whittet'. The four remaining CA accessions do appear to differ genetically from the majority of CA accessions. All are present on golf

courses in southern California. Southern California may be more genetically diverse than other parts of California, or the four accessions may have been found there because more sampling was done in the southern California area.

The variability of these four accessions may be due to mutation, or invasion from an extra-national source such as Mexico, Africa, or Asia. Kikuyugrass can spread via seeds or vegetatively on turf equipment or shoes. It is possible that introductions were made to these golf courses from other courses in Asia, Australia, or Latin America. Mutation could also account for the variability shown in these four accessions. Caetano-Anollés (1999) found high mutation rates in vegetatively propagated bermudagrass. Although kikuyugrass can spread via seed, it is thought to predominantly spread through vegetative means (Mears, 1970; Wilen et al., 1995; Gross, 2003). Kikuyugrass may also have a significant enough mutation rate to produce genetically diverse accessions.

The germplasm from the two collection groups offer some insight into genetic variation in Hawaii. Most accessions sampled from the Dr. Urata's collection clustered together. This is reasonable since the plots have not been maintained and perhaps became homogenized over time. While most cluster in CL 3 few others do show up in CL 2 and CL 1 indicating that the collection still maintains some diversity. It is not surprising that a few clustered with the CA accessions since CA was the source of parental lines in some instances. Of greater interest are samples taken from around the island of Hawaii. These clearly show there is genetic diversity in the wild kikuyugrass in Hawaii in natural stands. Such diversity is present even in a confined space, as illustrated by four samples from a pasture at the Mealani Experiment Station. In Hawaii,

kikuyugrass grows from sea level up to 1830 m (Fukumoto and Lee, 2003). The resulting varying environments may have aided in either mutation rate or more successful invasion events that added genetic variation to wild kikuyugrass in the state. The variation in the state was enough to aid in creating a new forage cultivar in 1983 (Fukumoto and Lee, 2003). This makes Hawaii a valuable potential resource for kikuyugrass collection.

Of the three major sources tested, The AUS germplasm showed the most diversity with a significant number of accessions in each of the three major clusters. This was expected since the AUS germplasm collection is not only the largest and best recorded collection but also the oldest. Of the 35 accessions from NSW only five were found in CL 2 or CL 3. This suggests a low overall amount of genetic variation within the state, although the rate is higher than what is seen in CA. Several of the accessions from NSW were recorded as having unique phenotypes: PM32 (shade tolerant), PM54 (cold tolerance and male sterile), PM58 (natural dwarf habit), PM59 (male sterile and very little lateral spread). PM45 is salt tolerant and locates in CL 2; it is genetically distant from the other accessions with noteworthy phenotypes. A similar situation occurred with the UCR accessions when they were first selected and unique growth characteristics were evident (e.g., fine leaf texture, dense growth habit) but the current genetic analysis shows low variation among UCR accessions (Cockerham et al., 1992). This indicates that similar accessions can still express unique phenotypes that may be useful in creating a turf-type cultivar.

The three accessions that are chemically induced mutants (PM49, PM50, and PM51) do not appear to be closely related to the original 'Whittet' from which they are allegedly derived. On the other hand, both selections from 'Whittet', AZ-1 and PM64, are in the same cluster as 'Whittet'. This suggests that either the mutagenesis process significantly modified the genome in all three accessions (PM51 especially), or that the original plant material may not have been the cv. Whittet. All that is known about the production of these three accessions is that they were created using chemical mutagenesis. If ethyl methanesulfonate (EMS) was used it could have induced enough mutations for PM49, PM50, and PM51 to cluster away from 'Whittet' (Morris, 2009; Sikora et al., 2011).

This study included four accessions that are considered to be the original inductions to Australia from east Africa, made in 1918 (PM28, PM60, PM86, PM87). All four cluster in CL 1 with most of the CA and AUS accessions, suggesting that most of what is currently growing and grown in CA and AUS is related to these initial introductions. Records indicate that California had two introductions: one by the USDA, possibly from Africa, and the other from the University of Pretoria in AUS (Garner, 1925). Younger et al., (1970) theorized that two or three accessions were originally introduced into CA which then cross hybridized to create CA 'common' or wild kikuyugrass. This pathway may account for low genetic diversity among California accessions. Australia has had multiple importation events and a much more diverse climate, both of which may have added to increased genetic variation (Morris, 2009).

Multiple cultivars of kikuyugrass have been developed in AUS. Three of these were included in past studies as well as this one. Previous molecular studies separated kikuyu into two broad groupings, with one group containing 'Whittet', 'Noonan', and some naturalized AUS lines. The other group consisted of six naturalized populations from old-established fields (Holton et al., 2007). That study was based on forty polymorphic loci. Another study by Morris (2009) based on 195 markers showed a more detailed picture of kikuyugrass cultivar relationships. He found a close relationship between 'Breakwell' and 'Noonan' but only a distant relationship between the two and 'Whittet'.

With circa 16,000 SNP markers it seems safe to assert that this study provides more insight into these relationships. The hierarchical plot in Figures 4.3-4.5 show that 'Breakwell' (PM47), 'Noonan' (PM90), and 'Whittet' all cluster closely in CL 3. Only one selection from 'Noonan' (PM71) was located in CL 2. This means that all cultivars, including the Arizona derived AZ-1, have little genetic difference among them, a sensible assertion given their origin. The cv. AZ-1 is a selection of 'Whittet', while 'Noonan' is a cross between 'Breakwell' and 'Whittet'(Cook et al., 2005). Low diversity between 'Whittet' and 'Breakwell' is more difficult to explain. One is said to come from selections made from germplasm brought to AUS from Kenya ('Whittet') while the other is a selection of a naturalized population that is thought to come from the Democratic Republic of the Congo (Cook et al., 2005). The two original introductions into AUS may have come from genetically similar germplasm in Africa, although this seems unlikely.

A more plausible explanation is that they may have intercrossed when they were both being developed at the Grafton Experiment Station in NSW, Australia.

The UCR accessions showed little genetic diversity. The absence of UCR accessions in CL 1 suggests that these accessions have not mixed with other CA germplasm since their outdoor planting 24 years ago. It is also worth noting that the UCR accessions C1, C11, and C17 were originally collected in CA to act as controls when rating the new UCR accessions. Even though they originated in CA they appear to have either hybridized with the other UCR accessions or been invaded by them since they do not cluster with the other CA accessions.

The hierarchical plot (Fig. 4.6) and Table 4.3 show that the UCR accessions did not remain homogeneous over the past 24 years. If that had been the case then each of the five samples taken over the three replicates would have all clustered together for each accession. At least one accession (K171A-D) had four of the five accessions sampled cluster together, but for most accessions it is apparent that some mixing has occurred. This should serve as a warning for all collections; proper care of the plots is essential if the accessions are to retain their genetic identity.

Vegetative invasion appears to be the most probable cause for mixing between some of plots (ex. K239A/B and K130A/B). These two plots are next to each other and rhizomes or stolons could have grown into the neighboring plots. Other invasions that were 10 or 15 m away are unlikely to have occurred via rhizomes or stolons. The most likely contributor to invasion between plots was mowing. Kikuyugrass invasion often occurs through management equipment (Gross, 2003). Since the UCR accession plots

were all mowed with the same reel mower on a weekly basis this seems the most likely cause for the mixing.

There is evidence that in addition to mixing, mutation has also taken place in the UCR accessions. In both C17A-D and K163A-D a branch length difference can be seen between the sub-clades that represents individual plots. The difference in branch length between two plots that are exclusively in their own clade together suggests that one of the plots has mutated to be genetically different from the other. If the differences were due to invasion or hybridization then other accession would be present in the clade.

Further research should be focused in three directions. Phenotyping and selection should begin on the germplasm from CA and UCR. These two germplasm sources are genetically different from each other. They are immediately available in CA and there are no restrictions on improving this material. Once desired phenotypes in each germplasm collection are identified then crossing between germplasm groups can begin.

Collections from around the island of Hawaii were more diverse than was originally expected. This diversity could be a major asset to breeding a new turf-type cultivar in CA if importation of live material to the mainland is permitted. It would be of considerable interest to sample all islands in the state and create a new collection to be maintained on one of the islands, for future breeding needs. Bringing in live kikuyugrass samples from Hawaii should be easier than from international sources.

Efforts should still be made to import kikuyugrass germplasm from Australia. Although multiple hurdles stand in the way of this it has been done before (Cockerham et al. 1992). Some restrictions would have to be waived from the Animal and Plant Health

Inspection Service before germplasm can be brought in from Australia and other countries. The Australian collection offers the greatest variation and institutions there have already bred and released new cultivars. Access to turf-type cultivars, mutated lines, and naturalized African germplasm could greatly aid the effort in creating a turf-type cultivar for California.

Significant genetic diversity exists within the accessions available in CA to begin breeding a turf-type cultivar. Germplasm collections do not cluster by geographic area. The accessions maintained at UCR have intermixed significantly since their original planting and should be evaluated and replanted. Focus should be on crossing accessions from the UCR accessions and accessions collected throughout the state. Both Hawaii and Australia are valuable potential repositories for kikuyugrass genetic diversity. Genetic diversity was broadest in the Australia collection. Despite the difficulty of importing plant material from both areas it should nevertheless be attempted since both offer wide diversity that could substantially aid in creating new cultivars for CA.

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Figures

Figure 4.1. The correct number of clusters, *K*, was estimated via the gap statistic. In the left panel, the blue and red curves are the estimated expectation of log (W_k) and the observed log (W_k), respectively. The right panel is the gap statistic plot. The number of clusters is set to range from 1 to 6. Created using 16,000 genome-wide SNP loci. The inferred optimal *K* is the elbow point in the left panel, which is indicated by the maximizing gap on the right panel. The gap curved is plotted in the format of Gap (K) ± standard deviation of log (W_k). The gap statistic gives the optimal number of clusters at 3.

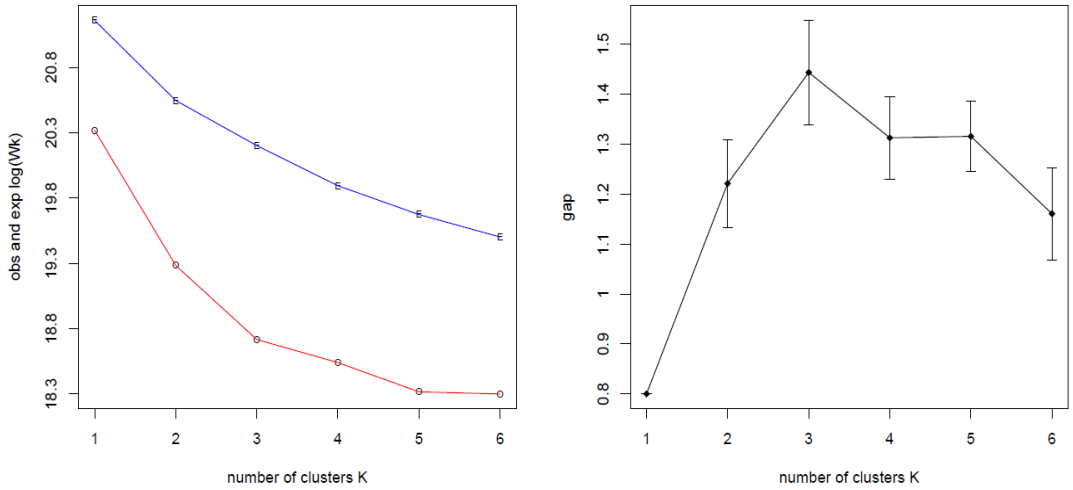
Figure 4.2. Principal Coordinate Analysis of all 336 accessions. Axis 1 explains 32.7% of the total variation. Axis 2 explains 3.4 % of the total variation.

Figure 4.3. Cluster 1 of hierarchical plot for all 336 accessions. CA- collected in California; PM- collected in Australia; HI- from Hawaii collection at the Mealani Research Station; HW- collected around the island of Hawaii. The red line passes through the hierarchical plot at the optimal number of clusters (K= 3).

Figure 4.4. Cluster 2 of hierarchical plot for all 336 accessions. CA- collected in California; PM- collected in Australia; HI- from Hawaii collection at the Mealani Research Station; HW- collected around the island of Hawaii; K and C- University of California, Riverside accessions. The red line passes through the hierarchical plot at the optimal number of clusters (K= 3).

Figure 4.5. Cluster 3 of hierarchical plot for all 336 accessions. CA- collected in California; PM- collected in Australia; HI- from Hawaii collection at the Mealani Research Station; HW- collected around the island of Hawaii; K and C- University of California, Riverside accessions. The red line passes through the hierarchical plot at the optimal number of clusters (K= 3).

Figure 4.6. Hierarchical plot for UCR accessions. Each accession was replicated 3 times in the field. Five samples were taken from each plot (A/B rep 1, C/D rep 2, E rep 3). The red line passes through the hierarchical plot at the optimal number of clusters (K= 3). Branch height represents dissimilarity.



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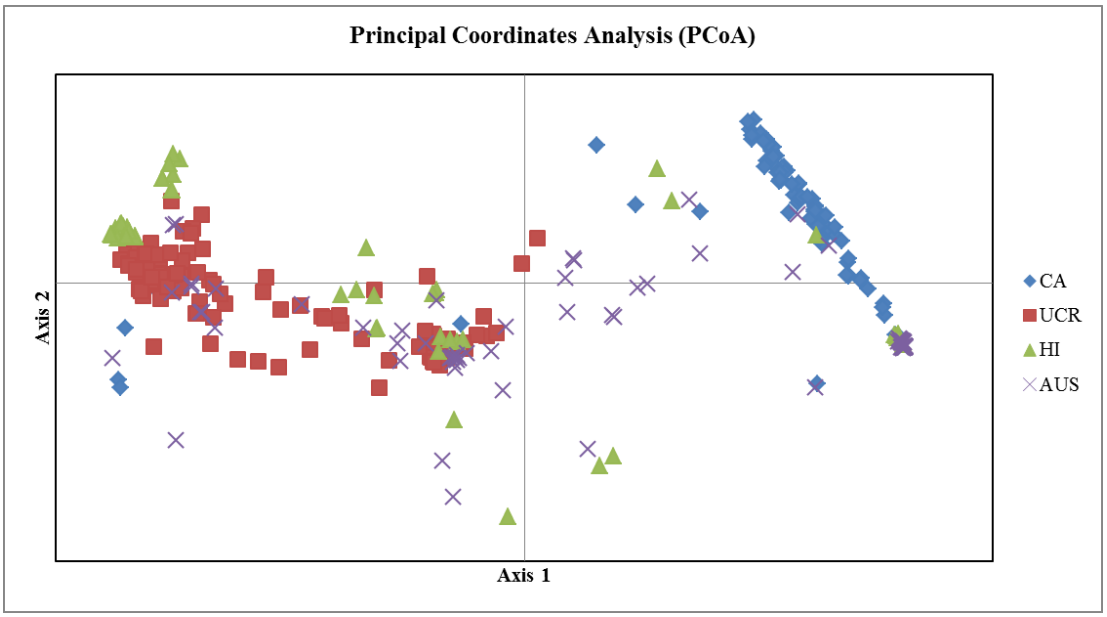

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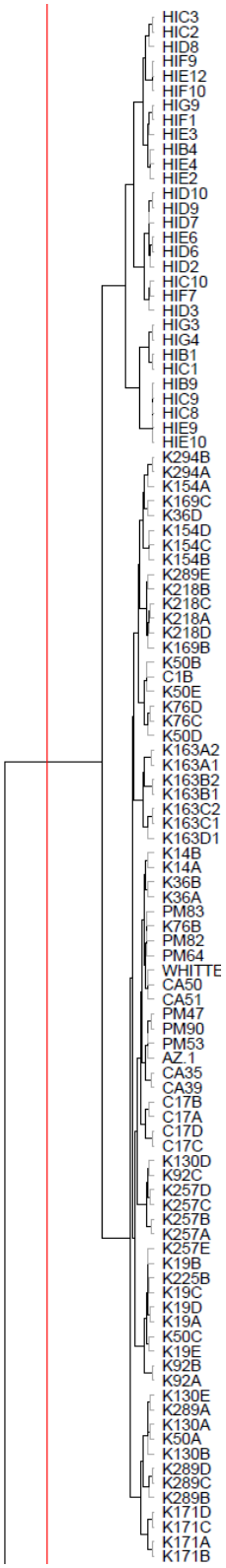
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HIF9
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HIF1
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HID9
HID7
HIE6
HID6
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HIC10
HIF7
HID3
HIG3
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HIC1
HIB9
HIC9
HIC8
HIE9
HIE10
K294B
K294A
K154A
K169C
K36D
K154D
K154C
K154B
K289E
K218B
K218C
K218A
K218D
K169B
K50B
C1B
K50E
K76D
K76C
K50D
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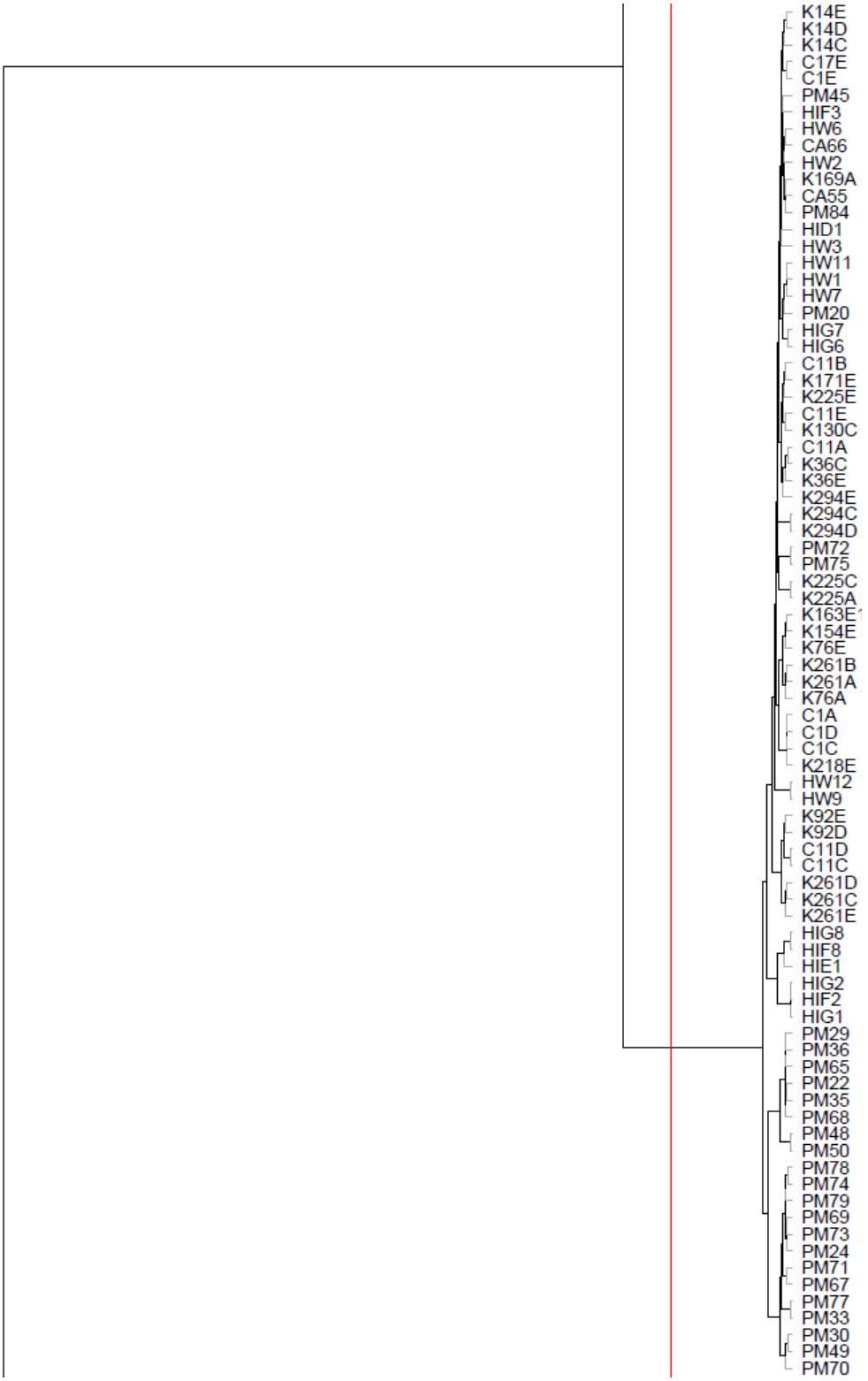
K163B2
K163B1
K163C2
K163C1
K163D1
K14B
K14A
K36B
K36A
PM83
K76B
PM82
PM64
WHITTE
CA50
CA51
PM47
PM90
PM53
AZ.1
CA35
CA39
C17B
C17A
C17D
C17C
K130D
K92C
K257D
K257C
K257B
K257A
K257E
K19B
K225B
K19C
K19D
K19A
K50C
K19E
K92B
K92A
K130E
K289A
K130A
K50A
K130B
K289D
K289C
K289B
K171D
K171C
K171A
K171B

Figure 4.5. Cluster 3 of hierarchical plot for all 336 accessions. CA- collected in California; PM- collected in Australia; HI- from Hawaii collection at the Mealani Research Station; HW- collected around the island of Hawaii; K and C- University of California, Riverside accessions. The red line passes through the hierarchical plot at the optimal number of clusters (K= 3). Branch length represents dissimilarity.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K14D
K14C
C17E
C1E
PM45
HIF3
HW6
CA66
HW2
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HW3
HW11
HW1
HW7
PM20
HIG7
HIG6
C11B
K171E
K225E
C11E
K130C
C11A
K36C
K36E
K294E
K294C
K294D
PM72
PM75
K225C
K225A
K163E1
K154E
K76E
K261B
K261A
K76A
C1A
C1D
C1C
K218E
HW12
HW9
K92E
K92D
C11D
C11C
K261D
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K261E
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HIE1
HIG2
HIF2
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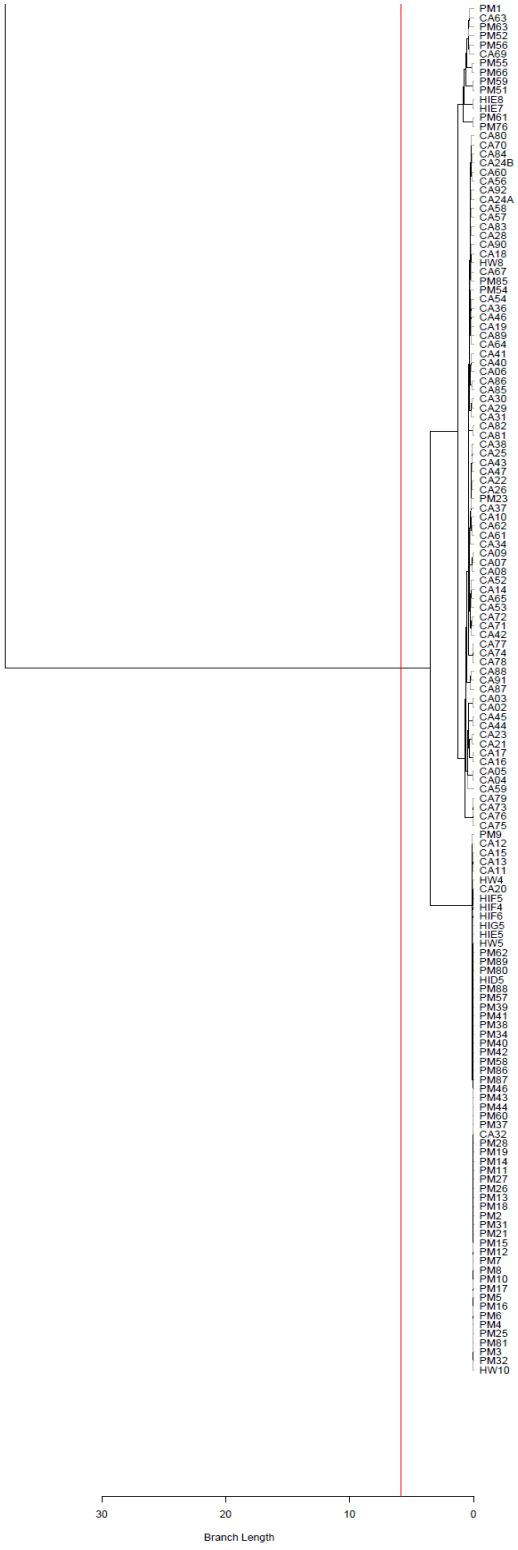
PM68
PM48
PM50
PM78
PM74
PM79
PM69
PM73
PM24
PM71
PM67
PM77
PM33
PM30
PM49
PM70

Figure 4.4. Cluster 2 of hierarchical plot for all 336 accessions. CA- collected in California; PM- collected in Australia; HI- from Hawaii collection at the Mealani Research Station; HW- collected around the island of Hawaii; K and C- University of California, Riverside accessions. The red line passes through the hierarchical plot at the optimal number of clusters (K= 3). Branch length represents dissimilarity.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CA11
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CA20
HIF5
HIF4
HIF6
HIG5
HIE5
HW5
PM62
PM89
PM80
HID5
PM88
PM57
PM39
PM41
PM38
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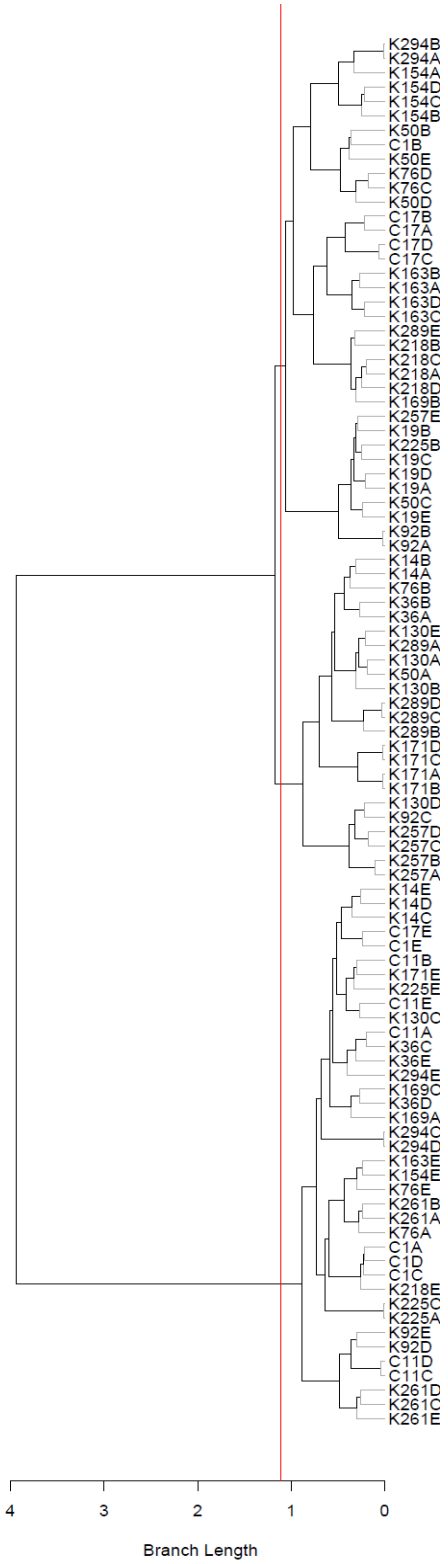
PM31
PM21
PM15
PM12
PM7
PM8
PM10
PM17
PM5
PM16
PM6
PM4
PM25
PM81
PM3
PM32
HW10
30
20
10
0
Branch Length

Figure 4.3. Cluster 1 of hierarchical plot for all 336 accessions. CA- collected in California; PM- collected in Australia; HI- from Hawaii collection at the Mealani Research Station; HW- collected around the island of Hawaii. The red line passes through the hierarchical plot at the optimal number of clusters (K= 3). Branch length represents dissimilarity.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K294A
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K154D
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C1B
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K76A
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C1D
C1C
K218E
K225C
K225A
K92E
K92D
C11D
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K261C
K261E
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Branch Length

Figure 4.6. Hierarchical plot for UCR accessions. Each accession was replicated 3 times in the field. Five samples were taken from each plot (A/B rep 1, C/D rep 2, E rep 3). The red line passes through the hierarchical plot at the optimal number of clusters (K= 3). Branch height represents dissimilarity.

Table 4.1. Analysis of molecular variance among populations and within populations for all 336 accessions.

Source	df	Ss	Est. Var.	% of total Variation
Among Pops	3	144851.3	558.4	23
Within Pops	332	605937.5	1825.1	77
Total	335	750788.9	2383.5	100

Table 4.2. Hybrid lines and their parents. The second parent is the pollen donor. Pollen donors came from the three mutated 'Whittet' accessions PM49, PM50, and PM51. Parents with KC numbers were not included in the diversity study.

Hybrid line	Parents
PM24	KC939 x PM49
PM35	KC924b x PM49
PM70	PM60 x PM49
PM72	PM39 x PM49
PM74	PM54 x PM49
PM78	PM8 x PM49
PM79	KC911 x PM49
PM36	KC944 x PM50
PM65	KC929 x PM50
PM67	KC908 x PM50
PM75	PM50 x PM50
PM76	PM61 x PM50
PM22	KC937 x PM51
PM33	KC9464 x PM51
PM48	KC947 x PM51
PM66	PM55 x PM51
PM68	PM31 x PM51
PM69	PM58 x PM51
PM73	PM57 x PM51
PM77	KC9465 x PM51

Table 4.3. Distances between mixed plots. Mixing was determined based on clustering in the hierarchical plot for UCR accessions (Figure 4.6).

Mixed accessions	Distance between plots
	meters
K36C/D and K169C/D	8.6
K130C/D and C11E	6.8
K92C/D and K130C/D	2.4
K19C/D and K50C/D	3.9
K50C/D and K19E	10.4
K225A/B and K50C/D	9.4
C1C/D and K50A/B	11.5
K19A/B and K14E	18.1
K289A/B and K130A/B	2.4
K50A/B and K130A/B	3.9
K169A/C and K218A/C	2.4
C1A/C and K50 A/C	14.6

Chapter 5

Overall Conclusions

Kikuyugrass management

Kikuyugrass is a turfgrass that is well suited to grow in California and will persist on golf courses, sports fields, parks and residential areas. In order to improve kikuyugrass in California three projects were carried out to look at improvements in management, cytogenetics, and plant breeding.

The management study found that increased mowing frequency on kikuyugrass increases turfgrass firmness but does not reliably reduce scalping or increase turf quality. Aggressive verticutting 2 times year^{-1} was better most seasons than a weekly grooming regime and increased turfgrass firmness, turf quality, color, and reduced scalping. The PGR trinexapac-ethyl had the most pronounced and positive effects on kikuyugrass ratings. The trend seen across all seasons except for fall 2012 and spring 2013 was that TE increased turf quality, color, ball roll, and reduced scalping.

Nitrogen fertilization treatments resulted in few significant differences; some results showed higher rates of N led to an increase in scalping but this trend was not continuous throughout years and seasons. Fungicides prevented damage from pathogens but did little else.

Androgenesis on kikuyugrass

Based on the results of the three androgenesis attempts, it is still unknown if androgenesis is a viable practice in reducing kikuyugrass vigor. It is also unknown if

androgenesis is even possible in kikuyugrass. No embryos were found and no mitosis was seen in microspores in any of the three attempts at androgenesis in either kikuyugrass or pearl millet. Unfortunately, these data do not prove conclusively that androgenesis is not possible in kikuyugrass. They simply show that these attempts were unsuccessful.

It is possible in the case of kikuyugrass that the whole species regardless of genotype is unsuited for androgenesis. This has been seen in onions, leeks, and many perennial and woody species. It is also possible that the common cause for the failed androgenic response in kikuyugrass and pearl millet was with the research execution. It could be that incorrectly prepared media, overstress of plant material, incorrect temperature regimes, or a host of other practices inhibited androgenesis in both species. The failure of both species to produce viable embryos makes pinpointing a direct cause difficult.

Genetic variation of kikuyugrass

Genetic variation exists among genotypes of all four germplasm collections. All 336 genotypes tested form three main clusters. These were seen in both the hierarchical plot and the PCoA, and were identified in the gap statistic plot. The variation between genotypes is not confined to geographic location. All four germplasm collections had individual genotypes in other clusters. Most importantly all germplasm collections had genotypes in CL1 and either CL 2 or CL 3. Only the UCR accessions had limited overall diversity with genotypes only in CL 2 and CL 3.

The CA collection both at UCR and from around the state as a whole showed the least variation. All but six CA genotypes were in CL 1. This reinforces previous findings of little genetic diversity among three golf courses in CA. The six genotypes that were genetically different from the rest of the CA genotypes were all found in southern California golf courses.

Germplasm from the two collection groups offer some insight into variation in Hawaii. Most of the genotypes taken from the collection at the Mealani Experiment Station clustered together. This is reasonable since the plots have not been maintained for many years. The samples taken from around the island of Hawaii proved to be very diverse. The diverse geography and climate in Hawaii, kikuyugrass grows from sea level up to 1830 m in Hawaii, may have aided in either mutation or more successful invasion events that added genetic variation to wild kikuyugrass in the state. Thus, Hawaii is a valuable potential resource for kikuyugrass collection.

The AUS germplasm showed the most diversity with a significant number of genotypes in each of the three major clusters. This was expected since the AUS germplasm collection is not only the largest and best recorded collection but also the oldest. Genotypes collected from different states in AUS showed the broadest diversity. All four of the varieties included in the study ('Whitter', 'AZ-1', 'Breakwell', 'Noonan') clustered close together indicating little diversity among them. The three genotypes that were created by chemical mutagenesis from the variety Whittet showed significant diversity from their source variety. Using chemical mutagens on kikuyugrass should be investigated as a potential tool for kikuyugrass improvement in CA.

The accessions maintained at UCR showed little genetic diversity. The lack of UCR accessions in CL 1 suggests that these accessions have not mixed with other CA germplasm since their outdoor planting 24 years ago. Homogeneity was not maintained across replications in the UCR accessions or even within most individual plots. The most likely contributor to invasion between plots was mowing. Mowers and other turf management equipment are known to facilitate kikuyugrass invasion.

Significant genetic diversity exists within the genotypes available in CA to begin breeding a turf-type cultivar. Focus should be on crossing genotypes from the UCR accessions and genotypes collected throughout the state. Both Hawaii and Australia are valuable potential repositories for kikuyugrass genetic diversity. Despite the difficulty of importing plant material from both areas it should nevertheless be attempted since both offer wide diversity that could aid in creating new cultivars for CA.